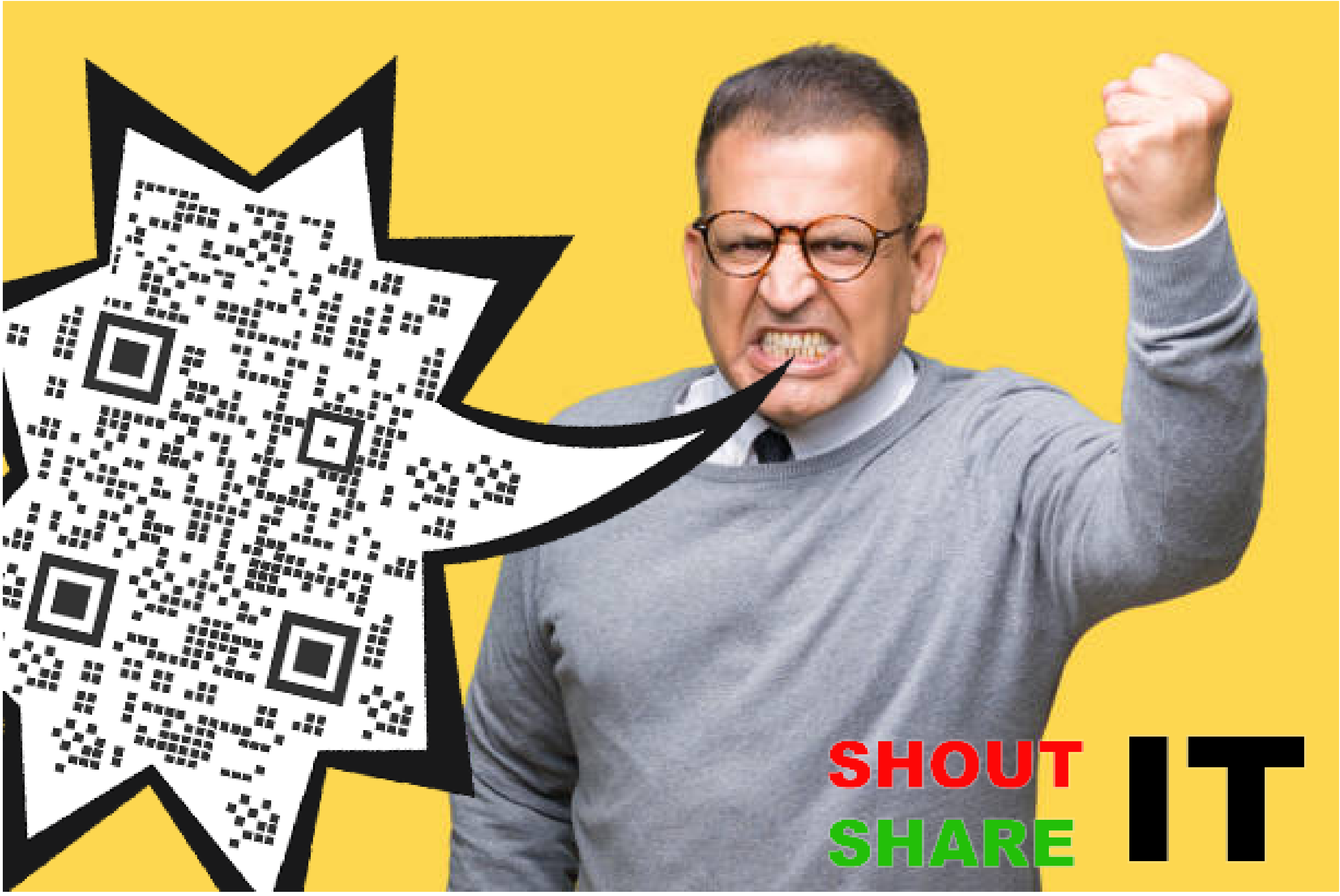
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THE

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ROLE OF THE CORPORA CARDIACA IN THE BEHAVIOR OF SATURNIID MOTHS. I. RELEASE OF SEX PHEROMONE

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Mating of Polyphemus moths under laboratory conditions requires the presence of a volatile emanation from oak leaves (Riddiford and Williams, 1967). The active material has been extracted from red oak leaves and shown to be *trans*-2-hexenal (Riddiford, 1967). Vapors of a dilute solution of this aldehyde were found to act upon the female antennae. The resulting nervous input to the brain provokes after a certain period of delay the "calling" behavior. The latter can be recognized by inspection in terms of the protrusion of the female genitalia thereby exposing the glands which emit the sex pheromone.

In the case of virgin Cecropia females, calling behavior is elicited, not by a chemical, but by photoperiod. Thus, under both long- and short-day conditions, calling begins 1.5 to 2 hours before dawn and often continues for as long as 0.5 hour after lights-on. During this same pre-dawn period, male Cecropia moths become hyperactive even in the absence of females.

The third silkmoth considered in the present study was *Antheraea pernyi*—a semi-domesticated species which, like the completely domesticated *Bombyx mori*, has for thousands of years been selected for ease of mating. Though virgin Pernyi females show no overt calling behavior under laboratory conditions, there is convincing evidence that the sex pheromone is continuously released to provoke mating at any time of day or night (Riddiford, 1970).

In the present study carried out on these three species we have sought to determine whether the corpora cardiaca or corpora allata are involved in the control of the release of sex pheromone.

MATERIALS AND METHODS

1. *Experimental animals*

Pupae of *Antheraea polyphemus* and *Hyalophora cecropia* were purchased from dealers or reared outdoors on netted trees (Telfer, 1967). Cocoons of *Antheraea pernyi* were obtained from Japanese sources. The pupae were stored

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at 5° C for at least 12 weeks; they were then returned to 25° C to provoke adult development.

2. *Excision of corpora allata and/or corpora cardiaca*

These organs were removed from pupae by the technique described by Williams (1959). To check the completeness of extirpation, the excised organs were placed in a black dish containing Ringer's solution and examined under a dissecting microscope. If the excised organs were not self-evident, the extirpation was considered incomplete and the animal was discarded. In certain individuals the glandular complexes were excised and three pairs of "loose" complexes reimplanted into the thoracic tergum. All individuals were placed at 25° C under controlled photoperiod (usually 17L:7D). Adult development was initiated after about 2 weeks and completed after an additional 3 weeks.

3. *Behavioral assays*

Female Polyphemus moths were caged in a darkened room and exposed to the vapors of 0.05% aqueous *trans*-2-hexenal solution. The experiment was usually begun in the early evening. At half-hour intervals for at least the first 4 hours, the moths were inspected under dim red light for calling behavior; they were again inspected the following morning.

In the experiments performed on Cecropia the female moths were reared and caged in two constant-temperature rooms, one programmed for a short day (12L:12D) and the other for a long day (17L:7D). Under dim red light the moths were inspected for calling behavior at hourly intervals throughout the scotophase.

In the experiments performed on *A. pernyi,* virgin females were caged with males and their mating behavior ascertained as described by Riddiford (1970).

EXPERIMENTAL RESULTS

1. *Delay in response of virgin female Polyphemus moths to vapors of trans-2-hexenal*

As described under Methods, 46 normal females were caged in a darkened room in the presence of the vapors of *trans*-2-hexenal. Observations under dim red light at 0.5 hour intervals indicated that at least one hour was required for the initiation of calling behavior and that 74% of individuals were calling after a total of 4 hours.

More detailed observations were carried out on a series of 9 virgin females which were placed, 1 or 2 at a time, in a 2-liter glass chamber in a darkened room. The chamber was ventilated by a gentle stream of air containing the vapors of a 0.05% aqueous solution of *trans*-2-hexenal. Observations of the moths were made at 15-minute intervals under dim red light.

The results summarized in Figure 1 once again show that at least an hour elapses before the first individual initiates calling. Fifty per cent of individuals initiated calling within the first 2.25 hours and 100% within the first 4 hours. From these observations we learn that a latent period intervenes between the

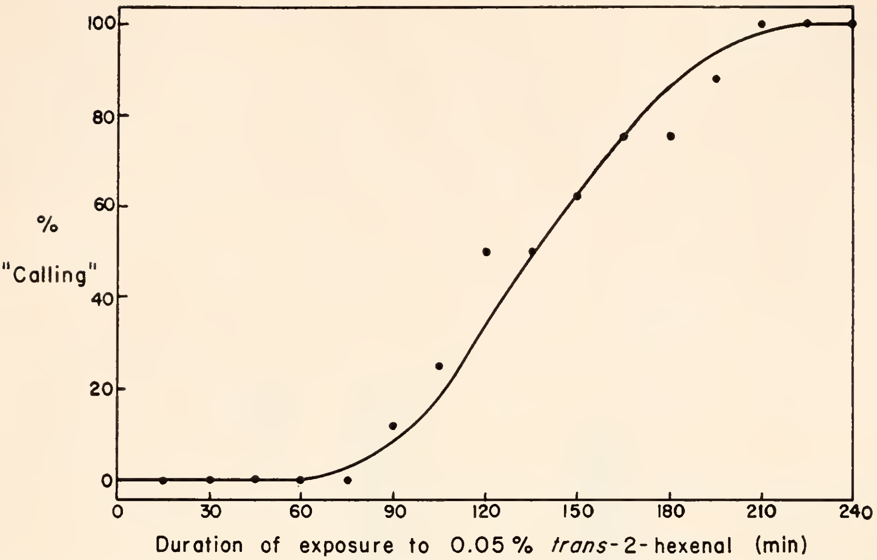

FIGURE 1. The time required for virgin Polyphemus females to begin releasing sex pheromone ("calling") after exposure to vapors of an aqueous solution of 0.05% *trans*-2-hexenal in the apparatus described under Materials and Methods. A total of 9 moths was used in these determinations.

presentation of the chemical stimulus and the initiation of the behavioral response. This delay was the first indication that a neuroendocrine relay mechanism might be involved.

2. *Effects of allatectomy*

The corpora allata were excised from 20 female Polyphemus pupae without any damage to the nearby copora cardiaca. The allatectomized pupae were then placed at 25° C and allowed to develop into adult moths. The latter's response to *trans*-2-hexenal was then determined as described under Methods. The results summarized in Figure 2 are the same as seen for unoperated Polyphemus moths. Moreover, the allatectomized females mated when placed with males and oviposited a normal number of eggs which hatched as normal first-instar larvae.

The experiment was repeated on 19 allatectomized Cecropia moths exposed to the short-day regimen of 12L:12D. Normal behavior was observed in terms of the presence of calling behavior during the final hour before lights-on. The results summarized in Figure 2 show that the absence of corpora allata in no way affected the response to photoperiod.

3. *Effects of removal of the complex of corpora cardiaca and corpora allata*

The experiment described in the preceding section was repeated on 29 Polyhemus and 38 Cecropia except that in this case the moths were derived from pupae lacking the entire complex of corpora allata and corpora cardiaca. The results summarized in Figure 2 show a great departure from normal behavior in that fewer than 20% of individuals showed calling behavior in response to the appropriate stimuli.

At the conclusion of the experiment many of the moths, including all individuals which had shown a calling response, were sacrificed. The heads were excised, pinned under Ringer's solution, and carefully inspected for any trace of the excised glands. The several individuals which showed any such indications were eliminated from the experiment. In Figure 2 the 14 to 18% of individuals which displayed the calling response showed no trace of corpora cardiaca. However, the dissection is a difficult one so there remains the possibility that the extirpation may have been incomplete in these individuals.

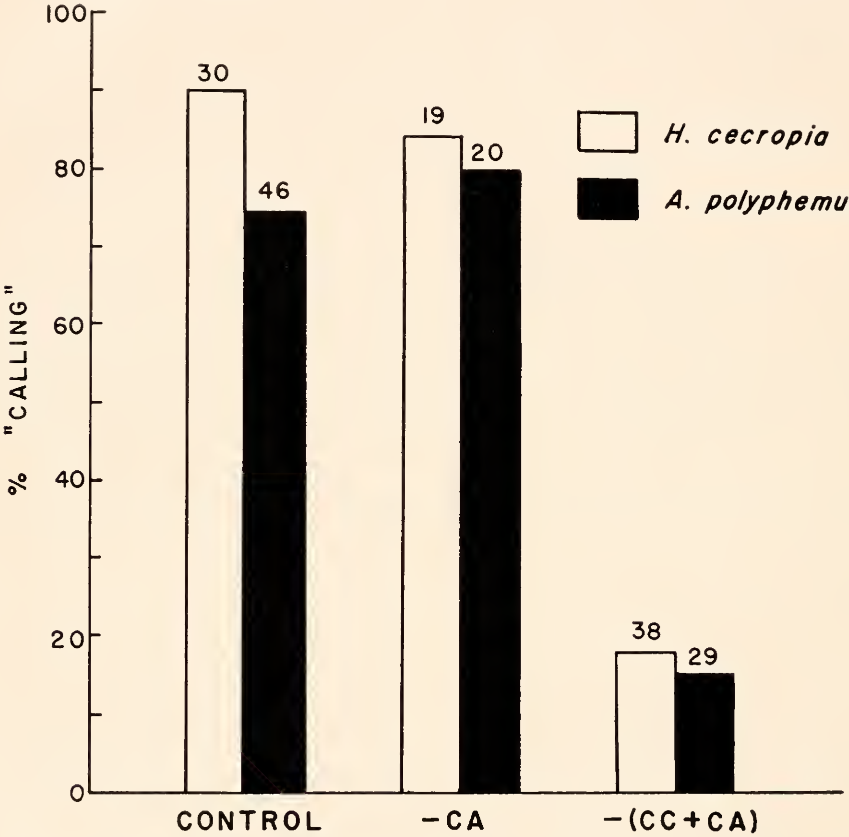

FIGURE 2. The effect of allatectomy and allatectomy-cardiactomy on the "calling" response of Polyphemus and Cecropia females to vapors of 0.05% *trans*-2-hexenal and to photoperiod respectively. The numbers in parentheses above the bars indicate the number of females tested.

4. *Re-implantation of the corpora allata-corpora cardiaca complexes*

The glandular complexes were excised from 11 female Polyphemus pupae and 2 female Cecropia pupae. Into the thoracic tergum of each individual were immediately reimplanted 3 pairs of "loose" glandular complexes. The moths derived from these preparations were tested for calling in response to the appropriate stimuli. The results were as follows: only 2 (18%) of the Polyphemus moths showed calling behavior when exposed to *trans*-2-hexenal vapors; none of the Cecropia moths showed calling behavior in response to photoperiod.

5. *Effects of denervating the corpora cardiaca*

In 5 female Polyphemus pupae the two pairs of nerves connecting the corpora cardiaca with the rear of the brain were severed. When the moths derived from these preparations were challenged with vapors of *trans*-2-hexenal, they showed no trace of the normal calling response. At the conclusion of the experiment autopsies performed on all 5 individuals showed no regeneration of the connections between brain and corpora cardiaca.

6. *Experiment on female Pernyi moths*

As mentioned in the introduction, *Antheraea pernyi* is a semi-domesticated species which has been highly selected for ease of mating. In experiments reported by Barth (1965) the allatectomized female Pernyi moths were fully effective in attracting and mating with males. The question therefore arises as to whether they can do so if the corpora cardiaca are also extirpated. To answer this question we excised the complex of corpora allata and corpora cardiaca from 16 female Pernyi pupae. The moths derived from these individuals were tested for the release of sex pheromone by caging them with normal males. Fifteen of the 16 females mated within 15 minutes, thereby documenting the continuous release of sex pheromone peculiar to the virgin females of this species.

Discussion

1. *The role of the corpora cardiaca in the reproductive behavior of virgin female silkmoths*

In contrast to the continous and apparently spontaneous release of sex pheromone by virgin females of semi-domesticated Pernyi silkmoths, the undomesticated Cecropia and Polyphemus silkmoths possess a neuro-endocrine mechanism for the control of pheromone release. Our experiments strongly argue that the corpora cardiaca, but not the corpora allata, are necessary for the calling behavior which accompanies the release of sex pheromone by the virgin female moths. That being so, the excision of the corpora cardiaca blocks the normal release of pheromone in response to environmental signals. This loss is not repaired by the reimplantation of as many as three pairs of "loose" glandular complexes—a finding which suggests that the nervous connections between the brain and corpora cardiaca are necessary for the behavioral response. When these nervous connections were selectively severed, calling behavior was blocked despite the continued presence of the denervated corpora cardiaca.

Females lacking corpora cardiaca fail to mate because males are not attracted to them. However, when caged with males near a cage of normal calling females, they not only mate, but then go on to lay fertile eggs. The absence of corpora cardiaca therefore interferes with the attraction of males but not with the ability to mate and reproduce.

2. *Dual role of the corpora cardiaca*

The corpora cardiaca serve as neurohaemal organs in which the products of the brain's neurosecretory cells are released into the blood. In addition, the corpora cardiaca contain intrinsic neurosecretory cells and therefore qualify as genuine endocrine organs. When the corpora cardiaca are excised, their intrinsic cells are permanently lost, whereas the neurohaemal portion promptly regenerates from the cut ends of *nervi corpora cardiaci* I and II (Stumm-Zollinger, 1957). So, for our present purposes it appears that the intrinsic cells of the corpora cardiaca are the source of the hormone which triggers the calling behavior of virgin females.

3. *The minimal circuitry*

In virgin Cecropia and Polyphemus moths the brain processes in-coming signals conveying specific environmental cues relating to the onset and timing of reproductive behavior. After this central integration, signals flow from the brain to the corpora cardiaca via the two pairs of nerves which interconnect them. On the basis of present knowledge we cannot say whether these signals are nerve impulses or neurosecretory agents. The signals in question converge on the intrinsic cells of the corpora cardiaca to provoke the release from these cells of a certain hormone. In the case of cockroaches, Milburn and Roeder (1962) have extracted from the corpora cardiaca a substance which causes rhythmic discharge in the phallic nerve when applied to the abdominal ganglia. Evidently, in the virgin moths an analogous factor is secreted by the intrinsic cells of the corpora cardiaca to promote the motor acts which comprise the calling behavior.

A similar type of mechanism involved in oviposition behavior will be examined in the further detail in the following communication (Truman and Riddiford, 1971).

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Summary

1. In virgin female silkmoths the protrusion of the genitalia or "calling" behavior signals sex pheromone release. The wild Polyphemus and Cecropia silkmoths "call" in response to specific environmental cues which are chemical and photoperiodic respectively. The semi-domesticated Pernyi silkmoth is exceptional in that it shows no overt "calling" behavior and pheromone is apparently released continuously.

2. By appropriate experiments it was possible to show that the corpora cardiaca but not the corpora allata are prerequisite for the "calling" behavior. Thus, when the corpora allata are removed from female pupae, the behavior of the resulting

moths is normal. By contrast, removal of the corpora allata-corpora cardiaca complex greatly reduces the number which "call."

3. In order to perform their function in the "calling" behavior the corpora cardiaca must have intact connections with the brain. Reimplantation of three pairs of corpora allata-corpora cardiaca complexes into animals lacking their own complexes fails to restore the ability to "call." "Calling" is also blocked when the nervous connections between the corpora cardiaca and the brain are severed.

4. Evidently, in response to the environmental signals the brain stimulates the release of a hormone from the intrinsic cells of the corpora cardiaca. This hormone then acts on the abdominal nervous system to provoke the protrusion of the female genitalia and the accompanying release of pheromone.

LITERATURE CITED

BARTH, R. H., JR., 1965. Insect mating behaviour: endocrine control of a chemical communication system. *Science,* **149**: 882–883.

MILBURN, N., AND K. D. ROEDER, 1962. Control of efferent activity in the cockroach terminal abdominal ganglion by extracts of corpora cardiaca. *J. Gen. Comp. Physiol.,* **2**: 70–76.

RIDDIFORD, L. M., 1967. *Trans*-2-hexenal: mating stimulant for Polyphemus moths. *Science,* **158**: 139–141.

RIDDIFORD, L. M., 1970. Antennal proteins of saturniid moths: their possible role in olfaction. *J. Insect Physiol.,* **16**: 653–660.

RIDDIFORD, L. M., AND C. M. WILLIAMS, 1967. Volatile principle from oak leaves: role in sex life of the Polyphemus moth. *Science,* **155**: 589–590.

STUMM-ZOLLINGER, E., 1957. Histological study of regenerative processes after transection of the nervi corporis cardiaci in transplanted brains of the Cecropia silkworm (*Platysamia cecropia* L.). *J. Exp. Zool.,* **134**: 315–326.

TELFER, W., 1967. Cecropia. Pages 173–182 in F. H. Wilt and N. K. Wessells, Eds., *Methods in Developmental Biology.* Thomas Y. Crowell Co., New York.

TRUMAN, J. W., AND L. M. RIDDIFORD, 1971. Role of the corpora cardiaca in the behavior of saturniid moths. II. Oviposition. *Biol. Bull.,* **140**: 8–14.

WILLIAMS, C. M., 1959. The juvenile hormone. I. Endocrine activity of the corpora allata of the adult Cecropia silkworm. *Biol. Bull.,* **116**: 323–338.

Reference: *Biol. Bull.*, **140**: 8–14. (February, 1971)

ROLE OF THE CORPORA CARDIACA IN THE BEHAVIOR OF SATURNIID MOTHS. II. OVIPOSITION

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Mating in the case of many insects serves as a pivotal event in the life of the adult female. This is especially true for the non-feeding Lepidoptera such as silkmoths of the family Saturniidae. In these insects one can define two conditions, the virgin state and the mated state, each with its own behavioral and physiological characteristics. A case-in-point is the silkmoth, *Hyalophora cecropia.* Here the virgin female assumes a "calling" posture for sex pheromone release at a specific time of the night (Riddiford and Williams, 1971). This behavior is terminated once mating takes place (Falls, 1933). Moreover, once mating has taken place oviposition is stimulated and longevity is reduced (Rau and Rau, 1914). Due to its quantitative nature we have chosen to study the rate of oviposition as an index of the change from the virgin to the mated state.

Materials and Methods

1. *Experimental animals*

The experiments were performed on the silkmoth *Hyalophora cecropia.* Diapausing pupae were obtained from Nebraska and were chilled for at least 12 weeks at 5° C prior to use.

2. *Excision of the corpora allata and corpora cardiaca*

The removal of the corpora allata or the corpora allata-corpora cardiaca complexes was performed as described by Williams (1959).

3. *Castrations*

Pupae were anesthetized with CO_2 and placed dorsal-side up in a plasticine cradle. A square of cuticle was excised from the dorsal midline of the third abdominal segment, and the underlying heart was pushed to the side of the operating field. The pupal testes, which are situated in the dorso-lateral region of the fourth abdominal segment, were removed with Dumont #5 forceps. A few crystals of phenylthiourea and streptomycin (Williams, 1959) were placed in the wound, and the operated area was covered by a piece of plastic cover-slip which was sealed in place with melted wax. To prevent the pupa from dislodging the seal, the fourth abdominal segment was immobilized by placing melted wax between segments 3 and 4. After 4 to 5 weeks at 25° C the castrated pupae gave rise to apparently normal moths which mated and produced well-formed, albeit sterile spermatophores.

4. *Matings of the moths*

The females were mated on the first or second day after their emergence. Unoperated females were placed with males in a 1 × 1 × 1 foot cage in the evening. The next morning non-mating animals were removed from the cage and stored elsewhere. The mating pairs were separated in the late afternoon.

In order to assure that all operated females mated, these females were hand-mated by a technique developed by C. M. Williams (personal communication). The female was deeply anesthetized with CO_2. An unanesthetized male moth was grasped by the wings and the genitalia were stimulated with a camel's hair brush to initiate the opening of the claspers. The tip of the female's abdomen was exposed by gentle pressure on the abdomen and brought into contact with the male genitalia. In most cases the males readily clasped the female and copulation ensued. The pair were separated 12 to 20 hours later.

The success of a mating to an unoperated male was judged by the production of fertile eggs. A successful mating to a castrated male was indicated by the remains of a spermatophore in the female's bursa copulatrix.

5. *Collection of eggs*

Each female was placed in a large paper bag. The number of eggs deposited each day was counted during each of the next six to seven days. At the end of this period, the females were sacrificed and dissected under Ringer's solution. The number of chorionated eggs remaining in the ovarioles was determined. This number was added to the total laid by each female and the latter expressed as a percentage of the total number of eggs that had matured.

In the graphs of the oviposition patterns of mated moths, the data are referenced to the number of days after the termination of mating. In the case of unmated females, the end of day 1 was scored when the first 5 or more eggs had been oviposited. In most cases this occurred the third or fourth day after emergence.

Results

1. *The effect of mating on the pattern of oviposition*

Figure 1A summarizes the cumulative per cent of eggs oviposited by 12 virgin females over a period of 6 days. Oviposition occurred at essentially a constant rate of 7% per day. After 6 days an average of 40% had been deposited and 60% retained.

To assess the effect of mating, 26 females were mated to unoperated males. Twenty-four of these crosses were fertile. The pattern of oviposition of these twenty-four is shown in Figure 1B. On the first day after mating the percentage of eggs laid by mated females was nearly as great as the total laid by virgin females over the entire six-day period. After six days, most females retained fewer than a dozen eggs in their ovarioles. This pattern of oviposition is essentially identical to that which Taschenberg and Roelofs (1970) reported for mated Ceropia.

The two unsuccessful matings were also of interest. Although the remains of a spermatophore were present in the bursa of both females, none of the eggs

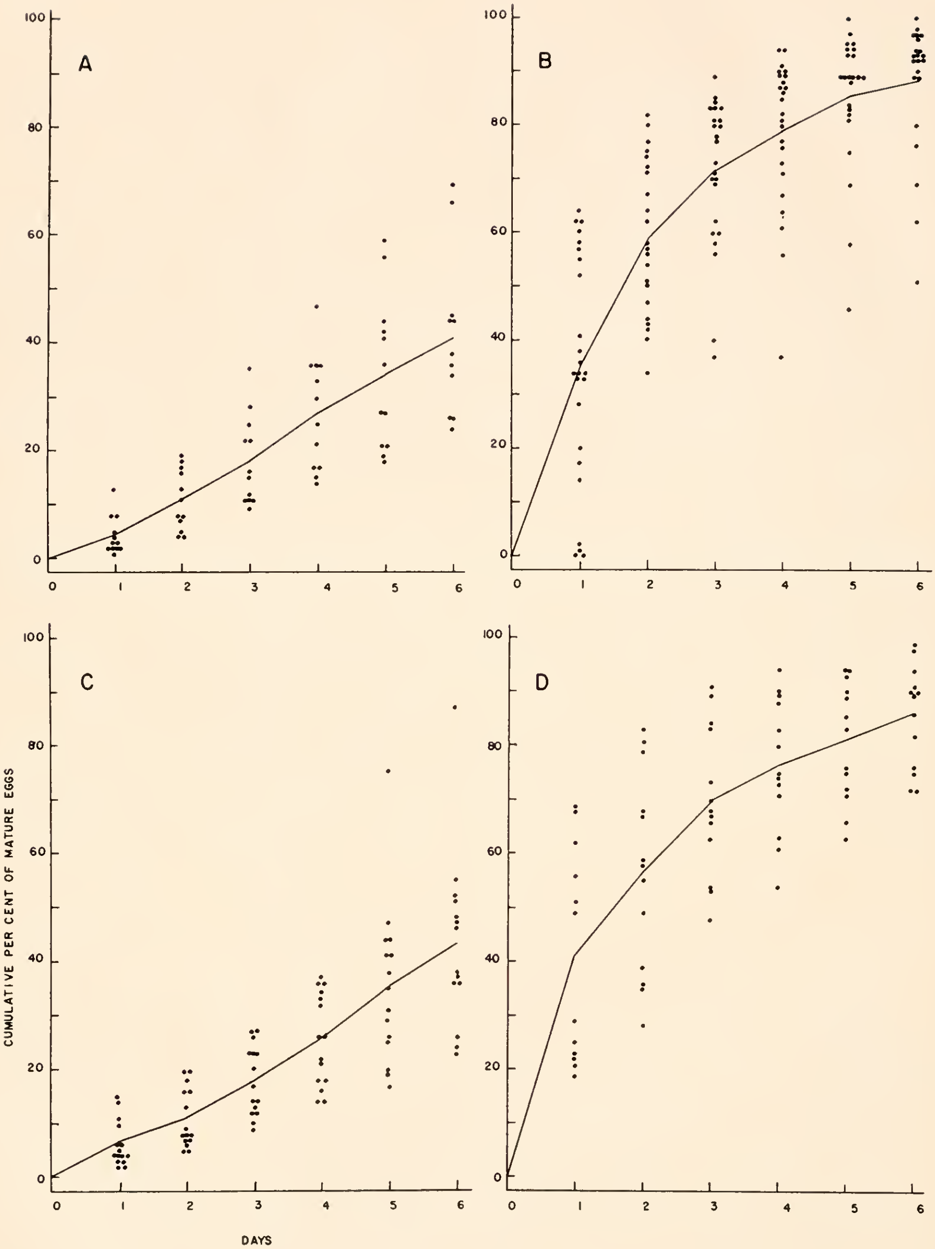

FIGURE 1. The oviposition patterns of Cecropia females: A., virgin females; B., females successfully mated to normal males; C., females successfully mated to castrated males; D., allatectomized females successfully mated to normal males.

was fertile. Both individuals showed a pattern of oviposition similar to that of virgin females.

2. *Matings to castrated males*

Fifteen females were mated to castrate males. As shown in Figure 1C, the pattern of oviposition was indistinguishable from that of virgin females. Upon autopsy, each female showed the remains of a spermatophore in the bursa copulatrix. Autopsy of the castrated males revealed normally developed accessory glands which were full of secretory products. Consequently, it was assumed that the spermatophores were normal except for the absence of sperm.

3. *Removal of the corpora allata*

The excision of the corpora allata does not interfere with oogenesis (Williams, 1959), or with the production and release of sex pheromone (Riddiford and Williams, 1971) by female Cecropia moths. However, there remained the possibility that the corpora allata might be involved in the switchover from virgin to mated behavior in response to insemination. To test this possibility the corpora allata were removed from thirteen female pupae. The pupae were then placed at 25° C and allowed to develop into female moths. The females were hand-mated to normal moths. As seen in Figure 1D, the removal of the corpora allata did not significantly interfere with the response of these females to insemination.

4. *Removal of the complex of corpora allata and corpora cardiaca*

The corpora allata-corpora cardiaca complexes were removed from 21 female pupae. Into the thorax of four of these pupae were reimplanted 4 or 5 pairs of "loose" corpora allata-corpora cardiaca complexes. A total of 14 of the resulting female moths were successfully mated to normal males (three of these had received implants of the complexes).

The removal of the complex of corpora allata and corpora cardiaca essentially abolished the response to insemination (Fig. 2A). As in the case of virgin females, the rate of oviposition was constant over a six-day period; the average rate was about 10% per day as compared to 7% for virgin females. The implantation of up to five pairs of complexes had no effect in restoring the mated response (Fig. 2A, open circles).

Seven females lacking corpora allata and corpora cardiaca were mated either to castrated males or to normal males without successful insemination. All seven showed the same type of behavior as unoperated females under the same conditions—*i.e.,* the virgin pattern of oviposition (Fig. 2B).

DISCUSSION

1. *The stimulus for the change in female behavior*

In most insects mating stimulates oogenesis and provokes an abrupt change in the behavior of the female (Norris, 1933). The specific stimuli which effect these changes are variable. In cockroaches the mechanical stimulus of the spermatophore in the bursa copulatrix brings about the mated response (Roth and Stay, 1961). In *Drosophila* (Kummer, 1960) and mosquitoes (Leahy and Craig, 1965) the transfer to the female of a secretion from the male accessory gland is responsible for the change. In the Lepidoptera (Klatt, 1920; Norris, 1933) and Hemiptera (Davey, 1965) the presence of sperm in the female spermatheca is apparently the trigger.

The present findings on Cecropia are in substantial agreement with those reported for other Lepidoptera. Matings with castrated males involved the passage of a sterile spermatophore but did not cause the switch in behavior. Consequently, the presence of sperm itself appears to be the stimulus which triggers the transformation of behavior to the mated pattern.

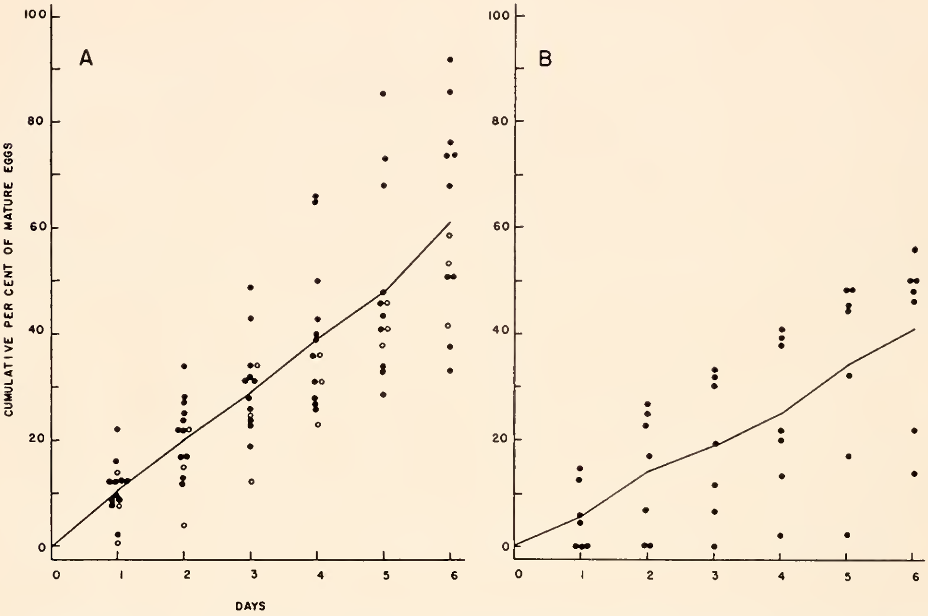

FIGURE 2. The oviposition patterns of allatectomized-cardiectomized Cecropia females: A., females successfully mated to normal males—the open dots refer to those which had 5 pairs of allata-cardiaca complexes reimplanted; B, females mated to castrated males or unsuccessfully mated to normal males.

2. *The endocrine relay: the corpora cardiaca*

The effect of mating on oogenesis is presumably mediated by the corpora allata (Engelmann, 1968). But in the case of the behavioral change, the involvement of the endocrine system has remained unexplored. The saturniids have proven to be convenient animals on which to study the change in oviposition behavior because in these insects egg maturation is independent of the corpora allata and the female moths emerge with essentially a full complement of eggs. Thus one circumvents any complications arising from a dependence of oviposition on oogenesis.

As seen in the Results, the corpora cardiaca, but not the corpora allata are necessary for the behavioral change. After removal of the corpora allata-corpora cardiaca complexes, mating resulted in only a slight elevation in the oviposition rate over that of virgin females. This was in marked contrast to the results obtained by mating unoperated or allatectomized females to normal males. The fact that the implantation of up to five pairs of complexes into cardiacectomized females did not restore the oviposition response shows the necessity of intact connections between the brain and corpora cardiaca. Therefore, the relationship is identical to that controlling pheromone release by these moths (Riddiford and Williams, 1971). The intrinsic cells of the corpora cardiaca apparently produce an oviposition stimulating factor, and the release of this factor is controlled by the brain.

It is important to note that the unsuccessful matings of allatectomized-cardiacectomized females gave the same result as seen in virgin females or in females which were mated to castrate males. From this we learn that the removal of the corpora cardiaca does not interfere with the process of egg-laying itself, but only with the increase in oviposition rate which is provoked by insemination.

3. *The role of the corpora cardiaca*

The corpora cardiaca appear to have a central role in controlling the behavior of the female moth. In the virgin condition the brain directs the release of a "calling" hormone from this organ (Riddiford and Williams, 1971). The results reported here indicate that after the reception of sperm by the female, the brain ceases to promote "calling" and now stimulates the release of an oviposition hormone from the corpora cardiaca. Consequently, the corpora cardiaca serve as the switch by which the brain changes the behavior of a female from the virgin to the mated condition.

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SUMMARY

1. In females of the Cecropia silkmoth, mating stimulates the rate of egg deposition.

2. When virgin Cecropia are mated to castrated males, they receive an apparently normal spermatophore except that it lacks sperm. Such females show an oviposition pattern corresponding to that of unmated females. Thus, the normal increase in oviposition rate is triggered by the reception of sperm by the female.

3. The corpora cardiaca are shown to be involved in this change in oviposition rate. After the corpora allata are removed from female pupae, the resulting moths respond normally to insemination. After removal of the corpora allata-corpora cardiaca complexes, the ovipositional response to mating is effectively abolished. Reimplantation of up to 5 pairs of corpora allata-corpora cardiaca complexes into pupae lacking their own complexes does not restore the normal oviposition pattern.

4. It is concluded that the increase in oviposition rate is due to a hormone which is produced by the intrinsic cells of the corpora cardiaca. By an apparent reflex mechanism, the presence of sperm in the female's spermatheca causes the brain to trigger the release of the hormone in question from the corpora cardiaca.

LITERATURE CITED

Davey, K. G., 1965. Copulation and egg-production in *Rhodnius prolixus:* the role of the spermathecae. *J. Exp. Biol.,* **42**: 373–378.

Engelmann, F., 1968. Endocrine control of reproduction in insects. *Ann. Rev. Entomol.,* **13**: 1–26.

Falls, O., 1933. Sex attraction in *Samia cecropia. Trans. Kansas Acad. Sci.,* **36**: 215–217.

Klatt, B., 1920. Beiträge zur Sexualphysiologie des Schwammspinners. *Biologisches Zentralblatt,* **40**: 539–558.

Kummer, H., 1960. Experimentelle Untersuchungen zur Wirkung von Fortpflanzungs-faktoren auf die Lebensdauer vor *Drosophila melanogaster* Weibchen. *Z. Vergl. Physiol.,* **43**: 642–679.

Leahy, M. G., and G. B. Craig, Jr., 1965. Accessory gland substance as a stimulant for oviposition in *Aedes aegypti* and *A. allopictus. Mosquito News,* **25**: 448–452.

Norris, M. J., 1933. Contributions towards the study of insect fertility. II. Experiments on the factors influencing fertility in *Ephestia kühniella* Z. (Lepidoptera, Phycitidae). *Proc. Zool. Soc. London,* **4**: 903–934.

Rau, P., and N. Rau, 1914. Longevity in saturniid moths and its relation to the function of reproduction. *Trans. Acad. Sci. St. Louis,* **23**: 1–78.

Riddiford, L. M., and C. M. Williams, 1971. Role of the corpora cardiaca in the behavior of silkmoths. I. Release of sex pheromone. *Biol. Bull.,* **140**: 1–7.

Roth, L. M., and B. Stay, 1961. Oöcyte development in *Diploptera punctata* (Eschscholtz) (Blatteria). *J. Insect Physiol.,* **7**: 186–202.

Taschenberg, E F., and W. L. Roelofs, 1970. Large-scale rearing of Cecropia (Lepidoptera: Saturniidae). *Ann. Entomol. Soc. Amer.,* **63**: 107–111.

Williams, C. M., 1959. The juvenile hormone. I. Endocrine activity of the corpora allata of the adult Cecropia silkworm. *Biol. Bull.,* **116**: 323–338.

Reference: *Biol. Bull.*, **140**: 15–27. (February, 1971)

LUMINESCENCE CONTROL IN *PORICHTHYS* (TELEOSTEI): EXCITATION OF ISOLATED PHOTOPHORES[1]

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All luminous fish are marine and the great majority of them live in deep waters. Consequently they are generally in poor condition after capture and not amenable to physiological study. An exception is the batrachoid teleost *Porichthys* (midshipman), a mid-water luminescent fish which moves inshore during the breeding season (Arora, 1948). It is well endowed with photophores, luminous spots on head and trunk, each containing a mass of photocytes (luminescent cells), nerve endings, a reflector, pigment-backing and a lens (Nicol, 1957; Strum, 1969). *Porichthys* is a hardy fish, easy to capture in good condition and to keep alive in captivity. These qualities make it favorable material for physiological investigations and, indeed, of the very few investigations of fish photophore physiology, the most extensive studies have been performed on this fish. It has been shown that *Porichthys* luminescence can be initiated by electrical stimulation of the whole animal (Greene, 1899), by injection of adrenaline (Green and Greene, 1924), or by electrical stimulation of the exposed spinal cord (Nicol, 1957). From these observations and a few others on deep sea fishes (Ohshima, 1911; Harvey, 1931), it is believed that photophores are either under hormonal control or nervous control, or that hormonal control is primary and nervous control secondary (Nicol, 1967). However, nothing is known either about the physiological properties of the photophore itself or about the manner in which the presumed control mechanisms initiate luminescence.

This work is devoted to the study of isolated *Porichthys* photophores subjected to electrical stimulation in order to analyze more precisely the excitation mechanism by elimination of all influences of the internal environment and to establish optimum conditions for further studies.

METHODS

1. *Maintaining fish and photophore preparations*

Specimens of *Porichthys myriaster* and *P. notatus* were collected in shallow water along the Pacific coast near Santa Barbara by divers or by dredging. After capture, they were placed in large aquaria with slowly running sea water. Before dissection, fish were removed from the aquarium and anaesthetized with quinaldine solution (25% in acetone) diluted to a final concentration of 3 ml per liter sea water, a concentration that induces anaesthesia after 3 to 5 minutes. Photo-

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[2] Chargé de recherches au F.N.R.S. (Belgium).

phores were then detached with as little surrounding tissue as possible. These experiments were all done on photophores from the anal, gastric and mandibular regions, as named by Greene (1899). All these respond similarly to electrical stimulation, although those of the mandibular region are the most consistently and readily excitable.

The isolated photophores, which are 1–2 mm in diameter, were placed in a small vessel filled with air-saturated Young's (1933) marine teleost saline (NaCl, 13.5 g; KCL, 0.6 g; $CaCl_2$, 0.25 g; $MgCl_2$, 0.35 g per liter solution). In order to adjust the pH to 7.4, the value recorded from blood samples, 1 mM $NaHCO_3$ was added.

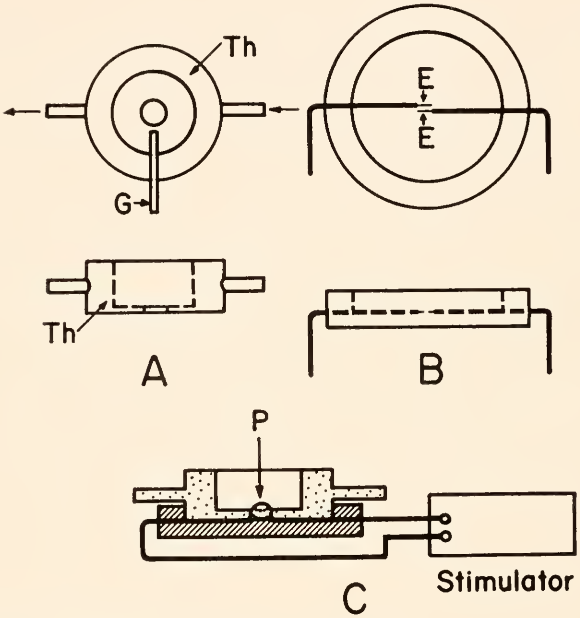

FIGURE 1. Recording chamber; A. thermostatted container (Th) through which a hypodermic needle (G) conducts gas to the specimen; B. plastic chamber bearing stimulating electrodes (E); C. the two chamber assemblies fitted together with a photophore (P) in position.

After dissection, fish were returned to the aquarium where they recovered after 15 to 25 minutes and could subsequently be used many times. Four specimens from which about 50 photophores were removed in this manner survived for 3 to 4 months. For the experiments reported here, 9 male specimens were used, of which 8 were specimens of *P. myriaster.* Their length ranged between 20 and 40 cm and their weights between 180 and 340 g.

2. *Recording chamber*

After being held for 10 minutes in saline at 20° C, the photophore was placed with dermal surface in contact with two platinum stimulating electrodes (diameter

100 μ) arranged in a chamber as shown in Figure 1. This is a double chamber: part A is a hollow cylinder 1 cm high, 2 cm inner diameter and 3.5 cm outer diameter, with a 4 mm diameter hole drilled in the base. A thermostated fluid flows through the space between the inner and outer walls. Part B is a lucite cylinder hollowed out in order to contain part A to a depth of 5 mm. Two grooves in the floor hold the electrodes. When the two parts are fitted together as shown in Figure 1C, the photophore protrudes into part A through the hole in its base and tissues surrounding the light organ are pressed between parts A and B, preventing movement of the specimen.

3. *Recording light emission*

A photomultiplier tube (RCA Type 1P21) was covered by a light-tight tube with an opening at the level of the photocathode. A ring was attached to the opening to accept the photophore chamber (Fig. 2). Thus the specimen could be enclosed with the light organ facing the photocathode at a distance of 15 mm. Oxygen, air, or nitrogen was passed into this enclosure through a small needle crossing obliquely the inner and outer walls of part A. Except for the nitrogen, the gases were humidified before reaching the photophore, which was stimulated in the gas phase.

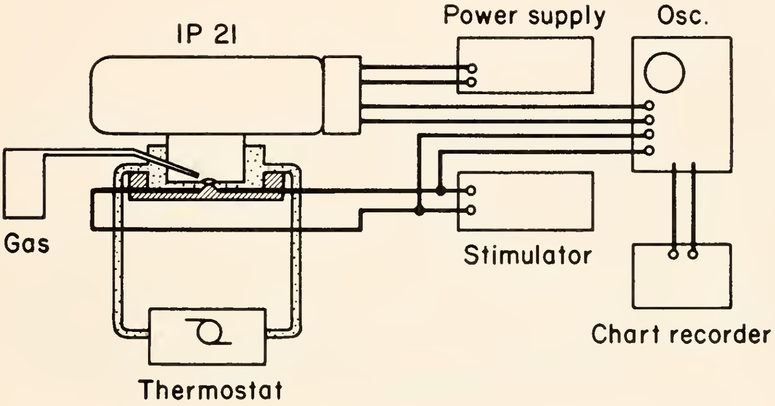

FIGURE 2. Diagram of apparatus used in detecting and recording luminescent responses to electrical stimulation.

Stimulating electrodes contacting the dermal surface of the photophore were connected to a Grass stimulator delivering square pulses. A stimulus artifact was registered by one of the two channels of a Tektronix 502A oscilloscope and one channel of a chart recorder (Offner Dynograph) connected in series to the oscilloscope. The luminescent response from the specimen activated the photomultiplier tube whose electrical signal was recorded on the second channel of the scope and the chart recorder. The photomultiplier was operated at 935 v delivered across a linear voltage divider. A quantitative estimate of light production was obtained with a standard light source in the same location as the experimental tissues. The standard was a disc covered with a C^{14} impregnated phosphor. The apparatus was generally used so that a 1 mm deflection of the chart recorder trace corresponded to 10^7 quanta/sec. Preparation resistance fluctuated between 400 kΩ and 900 kΩ.

RESULTS

1. *Types of light responses*

(*a.*) *Responses to single stimuli.* After 10 minutes in saline saturated with atmospheric oxygen, no light response was obtained with single stimuli applied by external electrodes. With microelectrodes (2–5 μ tip) inserted into the light organ itself, responses were obtained with single stimuli of 7 msec duration at 10 to 15 v. Figure 3 shows a recording of light emission after a 13 v, 20 msec stimulus (A) and a 10 v, 20 msec stimulus (B). The emission latency time, *i.e.*, the period between the stimulus and the first recorded light emission, ranged from 100 to 200 msec; the maximum light peak occurred after 0.6 to 1.0 sec and light disappeared after 4 to 8 sec.

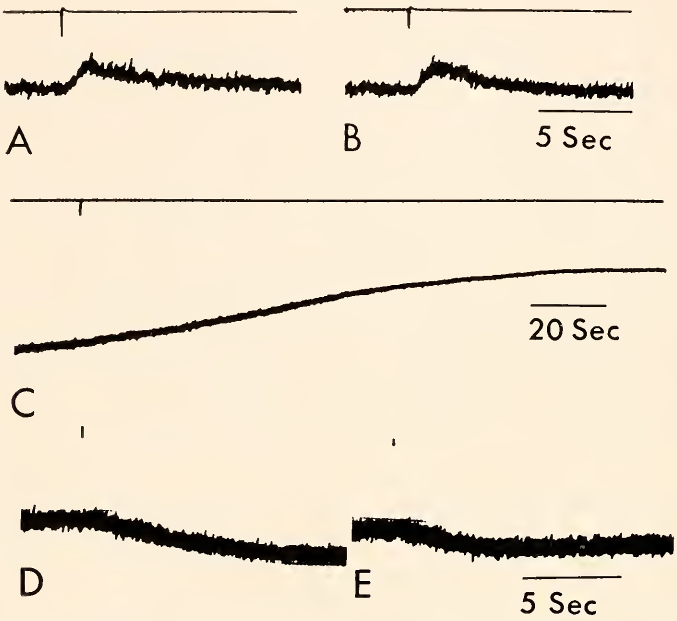

FIGURE 3. Light production of photophores responding to single stimuli; A and B with microelectrodes in photophore (A, 20 msec., 13 v; B, 20 msec, 10 v); C. a photophore stimulated with external electrodes (20 msec, 10 v) after 2 hours in saline; D and E. diminution of spontaneous glowing associated with stimulation by single pulses from external electrodes (7 msec, 10 v). A, B, D, E recorded at 5 mm/sec, C at 1 mm/sec.

The effect of a single stimulus, either *via* external or microelectrodes, was different for photophores which were bathed for longer than one hour in saline after dissection. Some specimens thus treated remained dark when placed in the chamber, while others spontaneously glowed. Those in the first category, when stimulated with single pulses, glowed for 20 to 30 minutes (Fig. 3C). Usually the photophore did not respond to the next stimulus, presumably a manifestation of injury. In spontaneously glowing specimens, light emission was reduced in a transitory way after a single stimulus. As shown in Figure 3D and E, the amplitude of the reduction depended on the strength of the stimulus: the effect was more evident at 10 v (D) than at 6 v (E).

It was generally found that all isolated photophores began spontaneously glowing after 2 to 3 hours in saline, and after a long-lasting light emission (30 to 60 minutes) became unexcitable electrically or chemically, undoubtedly consequent upon tissue deterioration.

(*b.*) *Responses to multiple stimuli.* In these experiments, electrical stimulation was delivered by external electrodes. Figure 4A shows a recording of typical light emission from a specimen stimulated with multiple stimuli, namely a 20 sec train of 7 msec, 8 v stimuli at a frequency of 10/sec (20°)C). A characteristically long emission latency time always occurred between the first stimulus and the first detectable light emission. Likewise, there was substantial delay, on the order of a second, between the last stimulus and the beginning of light extinction. Light emission, which was maximal after 21 sec, terminated about 130 sec after the end of stimulation.

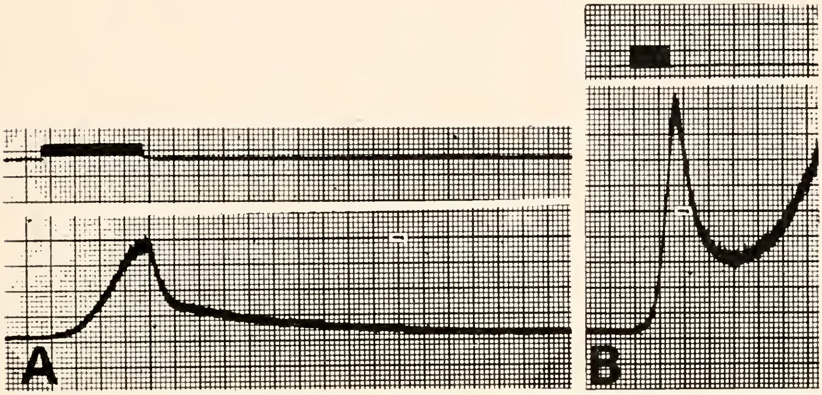

FIGURE 4. Photophore responses to repetitive electrical excitation; A. 20 second stimulus train at 10/sec, 7 msec, 8 v; B. 8 second train at 10/sec, 20 msec, 8 V; in both, photophore in oxygen atmosphere at 20° C; recording at 1 mm/sec.

For purposes of comparison with the effects of single shocks, it is evident that approximately 75 times more light is generated by the preparation shown in Figure 4A than is generated by the preparation of 3A.

With stimuli longer than 10 msec, spontaneous glowing occurred after the light response to electrical stimulation. The intensity of this glowing was about twice that of the initial response. The example shown in Figure 4B was induced by a 10 v, 20 msec, 10/sec stimulation for 8 sec. After such behavior, the photophore usually no longer responded to electrical, chemical, or mechanical stimulation. Consequently, in the experiments reported in this paper, 7 msec stimuli were used, permitting application of high frequency stimulation without spontaneous glowing which we assume to represent damage.

Repetitive bouts of stimulation initially cause an increase in response magnitude followed subsequently by diminished responses. This is illustrated in Figure 5 where emission rates (black circles) and the corresponding emission latency times (open circles) of 10 successive responses are plotted. At 10/sec responses became maximal at the fifth and subsequently diminished so that the response to the tenth stimulation was only 24% of the maximal. At 20/sec (Fig. 5B) the time course was faster and the response was maximal during the third stimulation while during the tenth the response was only 4% of maximum. The emission latency time was long during the first response, shortest when the response was maximal and thereafter increased again as the light response decreased in magnitude. The response was facilitated by stimulus train repetition, but after several trains fatigue appeared and light emission was reduced.

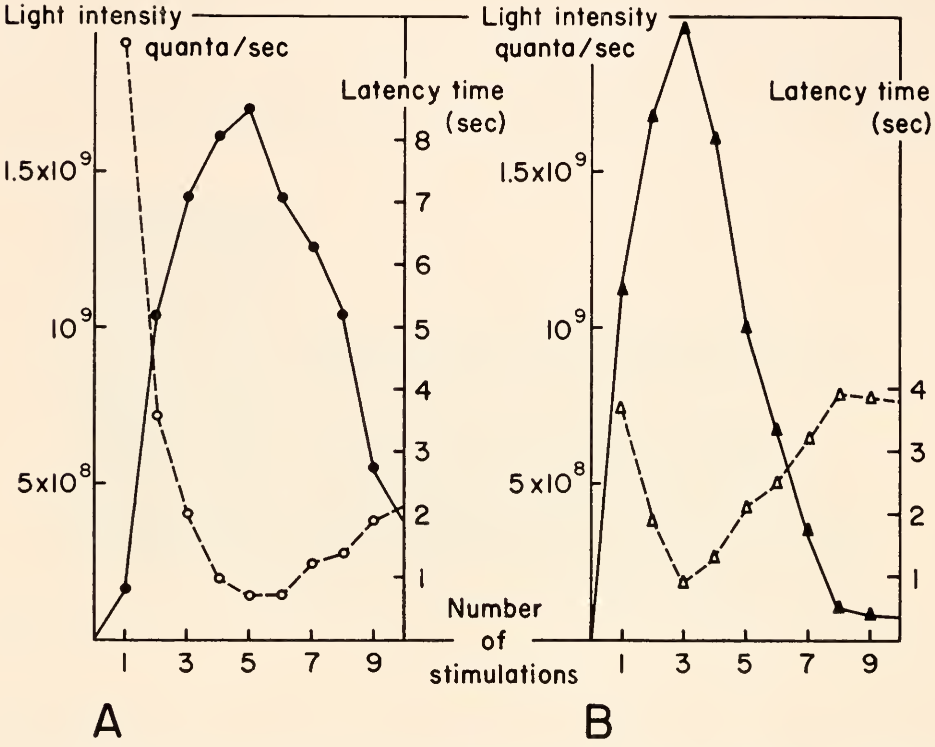

FIGURE 5. Emission latency (right ordinates) and rate of light emission (left ordinates) recorded during 10 stimulations of 20 seconds administered at 3 min intervals: A. 10/sec stimulation frequency, latency (○), emission rate (⊙); B. 20/sec stimulation frequency, latency (△), emission rate (▲).

2. Factors influencing light emission

(*a.*) *Electrical stimulation.* With external electrodes a minimal frequency of 4–5/sec was required to produce a response. The effects of higher frequencies were studied as follows: Each of a series of six photophores was stimulated for 20 seconds at 5, 10 and 20/sec and constant voltage (10 v). Because repetition facilitates the light response, the effects of different frequencies are influenced by the order of application. It was, therefore, necessary to randomize the treatments according to a latin square design (Cochran and Cox, 1957). Figure 6 shows the time course of the mean curves calculated for the three treatments. The magnitude of the light response increased with frequency: at 5 and 10/sec, light production increased during the entire stimulation period, while at 20/sec, the curve reached a plateau after about 15 seconds. At 5/sec the response was characterized by a long emission latency time (8.98 ± 1.18 sec, standard error of mean) and a low rate of light emission (7.3×10^7 quanta/sec at the peak). With an increase of frequency to 10/sec, emission latency time decreased significantly ($P < 0.01$) to 3.70 ± 1.18 sec and the rate of light emission was about 15 times greater (103.1×10^7 quanta/sec). At 20/sec, the latency time was shorter than that calculated for 10/sec (1.37 ± 1.8 sec) but the difference is not significant. On the other hand, the maximum rate of light emission was obviously

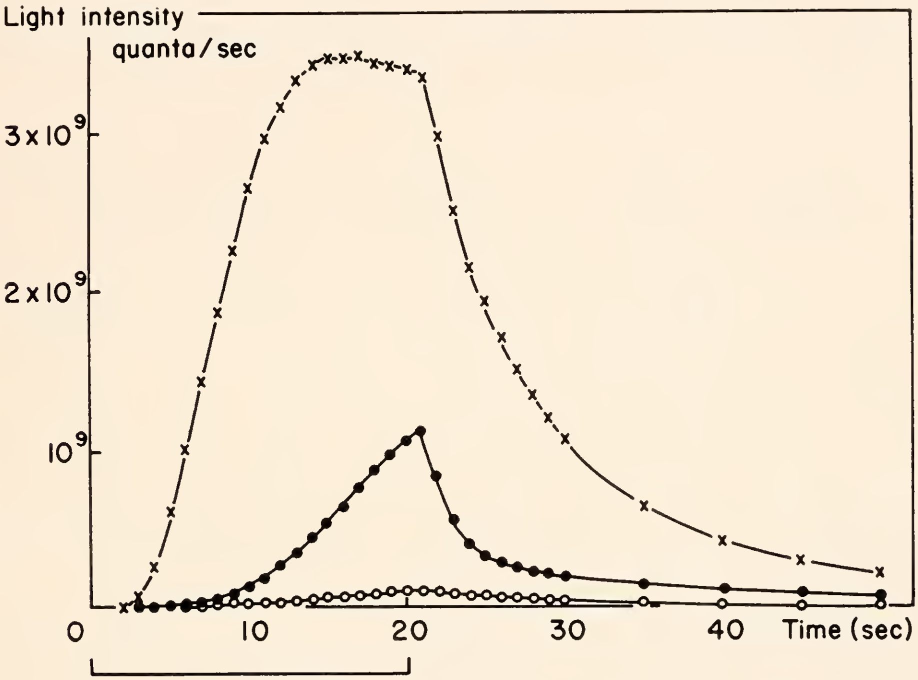

FIGURE 6. Light response of a photophore stimulated at three frequencies: 5/sec (○), 10/sec (●) and 20/sec (×). Horizontal bar indicates stimulus duration of 20 seconds. Prepared in 100% oxygen.

increased (335×10^7 quanta/sec). The half-extinction time; *i.e.*, the time necessary for the light to decline to half the peak value was 25.0 sec (5/sec); 23.9 sec (10/sec) and 28.3 (20/sec). With a standard error of the mean of ±0.7 sec, only the half-extinction time recorded at 20/sec was significantly different ($P < 0.05$) from those at 5 and 10/sec.

In all experiments, extinction was complete by 150 seconds after the end of stimulation. Total light produced during this period of time was obtained by integration: 207×10^7 quanta for a photophore receiving 100 stimuli (5/sec over 20 seconds), 1570×10^7 quanta for 200 stimuli (10/sec over 20 seconds) and 7490×10^7 quanta for 400 stimuli (20/sec over 20 seconds), standard error of mean 500×10^7 quanta. Thus at 20/sec, a photophore emitted about 5 times more light than at 10/sec. With higher stimulus voltages the shape and the magnitude of the light emission was not different from that recorded at 10 v.

TABLE I

Effects of saline pH on Porichthys *luminescence [Mean values calculated from 12 photophores stimulated in oxygen at 20° C (10 v, 10/sec, 20 seconds); each photophore treated at each pH in "cross-over" sequence; s.e.m. = standard error of mean]*

pH	Emission latency time seconds	Maximum light emission 10^7 quanta/sec	Extinction latency time seconds	Light production over 150 sec 10^7 quanta/sec
7.4	1.91	92.4	0.9	1,523.1
5.6	2.44	36.0	1.0	606.3
s.e.m.	±0.23	±12.1	±0.1	±179

The effects described above are observed only in cases where specimens are stimulated under specific physiological conditions; the photophore must be held in saline at pH 7.4 after dissection and be stimulated in oxygen at 20° C. The light response is modified when these conditions are not fulfilled.

(*b.*) *Saline pH.* Without adding 1 mM $NaHCO_3$, the pH of the saline was 5.6. When a photophore was stimulated after 10 minutes in this saline, it produced less light than after 10 minutes in the saline at pH 7.4 (Table I). At pH 5.6 the mean peak rate of light emission was only 36×10^7 quanta/sec while it was 92.4×10^7 quanta/sec at pH 7.4 ($n = 12$; standard error of mean $\pm 12.1 \times 10^7$ quanta/sec). The total amount of light produced was 2.5 times greater at pH 7.4 than at pH 5.6. As shown in Table I, neither emission nor extinction latency times were significantly changed by the change of pH.

(*c.*) *Oxygen pressure.* A reduction of the concentration of oxygen from 100% to atmospheric in the experimental chamber brought about a decrease in the light response: by 30% for a 10 v stimulation and by 50% for an 8 v stimulation. The other response characteristics did not change significantly.

When oxygen was removed and replaced by nitrogen for a period of between two and five minutes, the specimen was still excitable but the response was modified (Fig. 7). This figure shows the time course of the mean curve calculated for 8 photophores, each one stimulated, according to latin-square design, in oxygen and nitrogen at 8 and 10 v. The effects of anaerobiosis were an increase of emission latency time and a reduction of peak light production. The extinction phenomenon was not affected at 10 v but was significantly slowed at 8 v. Prolonged anaerobiosis was necessary to prevent the light response of a photophore. In all instances when a specimen was exposed to nitrogen for more than 5 minutes no light response was obtained on stimulation. However, it is significant to note that the stimulation itself changed the state of the light organ, for after stimulating in nitrogen readmission of oxygen led to spontaneous light emission lasting 2 to 5 minutes.

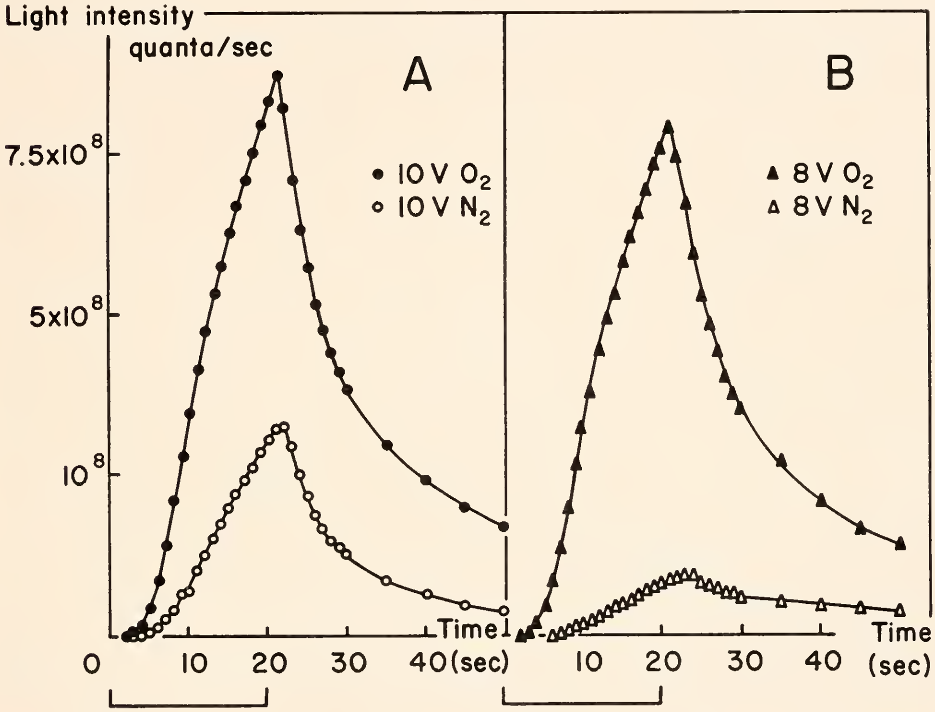

FIGURE 7. Mean curves of light production during stimulation at 10/sec for 20 seconds in oxygen and in nitrogen; A. 10 v; B. 8 v, temperature 20° C.

(*d.*) *Temperature.* Light emission was reversibly affected by temperature changes between 10 and 30° C, with the effects appearing 30 seconds after the temperature change. Figure 8 shows the time course of the mean curves of light emission at 10, 20, and 30° C ($n = 6$, 10 v, 10/sec, 20 seconds stimulation) with the three treatments randomized. Each specimen was held for 3 minutes at the experimental temperature in the thermostatted chamber before stimulation. The response was maximal at 20° C and minimal at 10° C. Table II summarizes the characteristics of the mean response were similar during and after the excitation period. At 10° C, the response was somewhat different; light appeared later [emission latency time significantly longer ($P < 0.01$)] and the magnitude

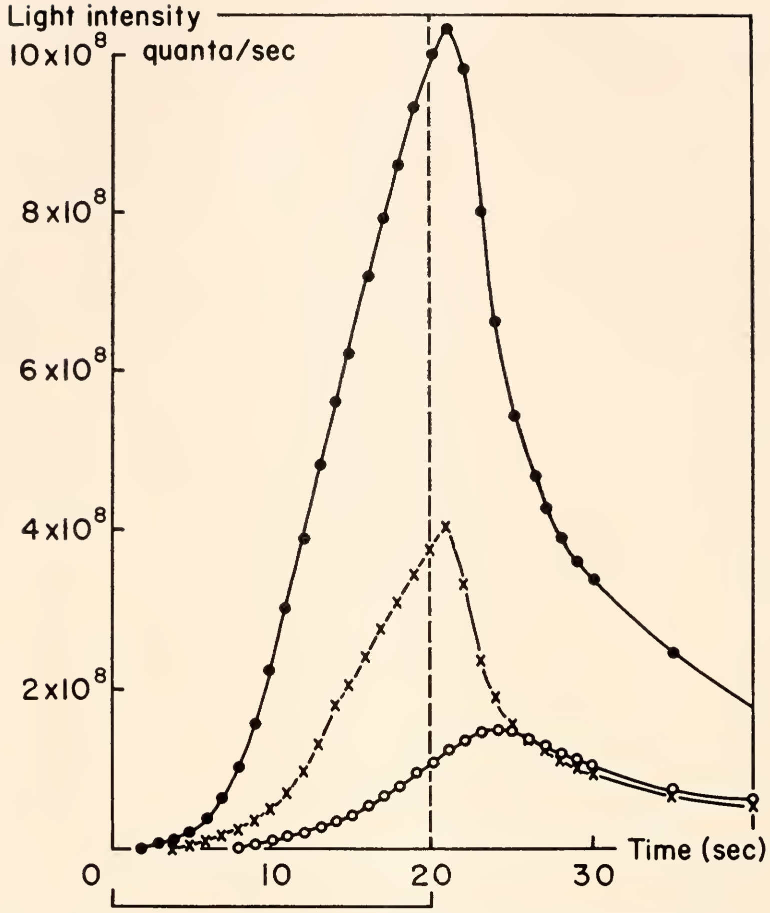

FIGURE 8. Mean curves of light production in response to stimulation at 10/sec for 20 seconds at 10° C (○), 20° C (●), and 30° C (×) in oxygen.

of the response was still increasing 5 seconds after the end of stimulation. Extinction was also significantly slowed ($P < 0.01$).

TABLE II

Temperature influence on Porichthys *luminescence ($n = 6$; in oxygen; 10 v, 10/sec, 20 seconds; s.e.m. = std. error of mean)*

Temperature °C	Emission latency time seconds	Maximum light emission 10^7 quanta/sec	Extinction latency time seconds	Half extinction time seconds
10	8.97	14.7	5.24	38.83
20	2.72	105.6	1.17	25.35
30	4.29	40.4	0.89	24.48
s.e.m.	±0.87	±10.5	±0.23	±1.98

Discussion

Isolated *Porichthys* photophores produce light upon electrical stimulation. Their response is characterized by a relatively long emission latency time, never less than one second, and by progressive increase in the rate of light emission during stimulation. If the emission latency time is the time required to gain a threshold at which the light response is triggered, then threshold is reached about three times at late at 5/sec as at 10 or 20/sec. However, since the total number of stimuli delivered during this latent period is the same for the three test frequencies, it is suggested that the effects induced by each stimulus are additive and that light appears when this sum reaches a threshold. As might be expected, if the interval between two stimuli is too long, no light emission is triggered, as seen at low frequency (2/sec). Similarly, Nicol (1957) observed that stimulation of the spinal cord of *Porichthys* with a train of pulses at frequencies below 3/sec was unable to induce luminescence.

In Nicol's experiments latency time varied from 7 to 15 seconds at 4/sec. Strum (1969), who studied nerve fibers of photophores by electron microscopy, suggests that the response time measured by Nicol corresponds largely to the time taken by the nervous influx to reach the photophore from the CNS. According to our data on the isolated photophore, the emission latency time varies from 5 to 19 seconds for a 5/sec stimulation. Thus photophores stimulated either *in situ* through the spinal cord or after isolation, at similar frequencies show a similar latency time. It is possible that, in our experiments, we have stimulated nervous elements isolated along with the light organ. If the electrical current stimulates nerves in our experiments, the latency time cannot correspond to nerve conduction time but rather might be associated with processes occurring at the level of "nerve-photocyte" junctions. Strum (1969) describes nerve-photocyte junctions having a specialized structure: non-myelinated nerve fibers enter the light organ from a deep subdermal plexus (Whitear, 1952) but do not make direct contact with the photocytes, but rather with a "basal lamella" surrounding the posterior regions of the photocytes. These are separated from the lamella by a sinus which appears to be empty. Finally, it should be recalled that when microelectrodes are implanted in the photophore, there is a response to a single pulse with a much shorter latency time, about 100 to 200 msec. The different properties of excitability and the short latency time thus apparent might imply that, in this case, the photogenic cells are directly stimulated. This is borne out by experiments in progress that show the minimal latency for light generation by photophores *in vivo* is in excess of 1 second for electrical excitation of the photophore nerve and not less than 5 seconds for nor-epinephrine injection within a millimeter of the photophore.

Once triggered, light production increases progressively during stimulation. According to Nicol (1957) a photophore contains about 600 to 700 photogenic cells. This progressive increase might correspond either to a recruitment of cells or, assuming all the cells are simultaneously stimulated, to an increase of their light emission. Our results do not enable us to distinguish between these two hypotheses.

The magnitude of the light response is particularly dependent on oxygen pressure and temperature. Light production on stimulation is diminished by a reduction of the oxygen pressure and it is suppressed when the photocyte is exposed to N_2 for more than five minutes. However, it is most interesting that anaerobiosis does not suppress the excitability of the light organ because on readmitting O_2 after stimulation in nitrogen, a spontaneous light response occurs. Stimulation may liberate reactants involved in the light reactions, but these possibly cannot react until oxygen is supplied. Certainly oxygen seems to be essential for these reactions to take place. Cormier, Crane, and Nakano (1967) extracted luciferin and luciferase from *Porichthys* photophores, and these were shown to react and produce light when in the presence of oxygen. The presence of numerous mitochondria in the photocytes (Strum, 1969) suggests that intensive oxidative reactions might take place in these cells.

The magnitude of the luminescent response is greatest around 20° C. The temperature coefficient (Q_{10}) calculated by comparing total light production at 10 and 20° C, is estimated as 4.5. The temperature coefficient of the light reaction itself is probably lower; according to Udenfriend (1962) the Q_{10} of chemoluminescent reactions is not greater than 1.5. Indeed the coefficient of the luminous system of the crustacean *Cypridina hilgendorfii in vitro* is estimated at 1.14 (Chase and Lorentz, 1945). There is no information concerning the effects of temperature on the isolated luminous system of *Porichthys*. The high value we calculate might be explained by assuming the presence of a series of reactions preceding the luminous reactions; depolarization phenomena, for example.

It is surprising that the optimal temperature for light production is around 20° C, when the temperature of the *Porichthys* mid-water habitat is around 10° C. Perhaps an explanation lies in the fact that during the spawning season, the fish seeks warmer water. Crane (1965) observed the courtship display of *Porichthys* in the aquarium and observed a well-defined associated pattern of luminescence. Since bioluminescence plays a role in the courtship pattern of *Porichthys* and since isolated photophores produce little light at low temperature (10° C), it is conceivable that the mechanisms of luminescence are better adapted to the breeding environment rather than to its usual habitat.

From our results, the light energy produced by a photophore may be roughly estimated, assuming the wavelengths of light from an intact photophore and from the luminous system *in vitro* are similar, approximately 460 $m\mu$ (Cromier, Crane and Nakano, 1967). For minimal stimulation at 10° C, the peak of light production is estimated as 6.5×10^{-1} microwatt; at 20° C, 4.6×10^{-4} microwatt. For a maximal stimulation at 20/sec and 20° C oxygen, the output is 1.4×10^{-3} microwatt. These computations are rendered highly approximate if only owing to the difficulty of ascertaining the geometry of light emission and the possibility of light absorption by associated tissues and especially chromatophores.

We are most indebted to Mr. Jules Crane, Dr. Richard Ibara and Mr. M. S. Trinkle for assistance in obtaining *Porichthys* and for their most helpful advice and assistance.

SUMMARY

Luminescene of isolated photophores of midshipman fish, *Porichthys myriaster* and *P. notatus,* was studied as a function of electrical stimulus characteristics, temperature, oxygen concentration and pH. Single stimuli delivered into the

photophore *via* microelectrode induced luminescence with a minimum latency of 100 msec. Externally applied stimuli were ineffective at rates of less than 2/sec and at 5/sec latency was between 5 and 19 seconds. These values suggest the possibility of both direct and indirect photophore excitability. Oxygen is essential for luminescence, but spontaneous occurrence of luminescence after electrical stimulation under nitrogen suggests that certain elements of the excitation-luminescence sequence are relatively insensitive to anaerobiosis. The temperature optimum is at about 20° C and the Q_{10} is 4.5. Total light produced was greater at pH 7.4 than at 5.6. Maximal light production is estimated at 1.4×10^{-3} microwatt per photophore.

LITERATURE CITED

Arora, H. F., 1948. Observations on the habits and early life of the batrachoid fish, *Porichthys notatus* Girard. *Copeia,* **1948**: 89–93.

Chase, A. M., and P. H. Lorenz, 1945. Kinetics of the luminescent and nonluminescent reactions of *Cypridina* luciferin at different temperatures. *J. Cell. Comp. Physiol.,* **25**: 53–63.

Cochran, W. G., and G. M. Cox, 1957. *Experimental Designs,* [2d. edition] J. Wiley, New York, 611 pp.

Cormier, M. J., J. M. Crane and Y. Nakano, 1967. Evidence for the identity of the luminescent system of *Porichthys porosissimus* (fish) and *Cypridina hilgendorfii* (crustacean). *Biochem. Biophys. Res. Commun.,* **29**: 747–752.

Crane, J. M., 1965. Bioluminescent courtship display in the teleost *Porichthys notatus. Copeia,* **1965**: 239–241.

Greene, C. W., 1899. The phosphorescent organs in the toad fish *Porichthys notatus* Girard. *J. Morphol.,* **15**: 667–696.

Greene, C. W., and H. H. Greene, 1924. Phosphorescence of *Porichthys notatus,* the California singing fish. *Amer. J. Physiol.,* **70**: 500–507.

Harvey, E. N., 1931. Stimulation by adrenalin of the luminescence of deep-sea fish. *Zoologica,* **12**: 67–69.

Nicol, J. A. C., 1957. Observations on photophores and luminescence in the teleost *Porichthys. Quart. J. Microscop. Sci.,* **98**: 179–188.

Nicol, J. A. C., 1967. The luminescence of fishes. *Symp. Zool. Soc. London,* **19**: 27–55.

Ohshima, H., 1911. Some observations on the luminous organs of fish. *J. College Sci. (Tokyo),* **27**: 1–25.

Strum, J. M., 1969. Fine structure of the dermal luminescent organs, photophores, in the fish, *Porichthys notatus. Anat. Rec.,* **164**: 433–467.

Udenfriend, S., 1962. *Fluorescence Assay in Biology and Medicine.* Academic Press, London, 106 pp.

Whitear, M., 1952. The innervation of the skin of teleost fishes. *Quart. J. Microscop. Sci.,* **93**: 289–305.

Young, J. Z., 1933. The preparation of isotonic solutions for use in experiments with fish. *Pubbl. Sta. Zool. Napoli,* **12**: 425.

Reference: *Biol .Bull.,* **140**: 28-45. (February, 1971)

THE CILIARY CURRENTS ASSOCIATED WITH FEEDING, DIGESTION, AND SEDIMENT REMOVAL IN *ADULA (BOTULA) FALCATA* GOULD 1851[1]

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Adula falcata, the pea pod shell, is a common byssally attached rock borer in soft mudstone reefs at Bolinas and Moss Beach, California (Fig. 1). While it possesses the protective advantage of living cryptically, *A. falcata* is subjected to an environmental stress not faced by most epifaunal mytilids. Namely, it must function within a burrow into which sediment is being continuously deposited from both particle-laden water passing over its burrow entrance and the mudstone byproducts of its own mechanical boring. This report is a comparative study on the ciliary mechanisms of feeding, digestion, and sediment removal in *A. falcata.* Sediment removal can hardly be considered separately from feeding and digestion, as it is during these later two processes that sediment is resolved from potential food material and extruded from the burrow.

Materials and Methods

A clear plastic mold of the burrow was constructed for observing the method employed in removing the sediment produced during mechanical boring. A piece of mudstone containing a live specimen of *A. falcata* within its burrow was cracked open and the boring mussel carefully removed. A soap model was made of the burrow and a transparent "Bioplastic" two-piece mold (dorsal and ventral pieces with respect to the orientation of the bivalve) was cast of the soap figure. The finished mold was washed in running seawater for several days to remove traces of water soluble chemicals present on the plastic's surface. The original living bivalve, was naturally positioned in the plastic burrow and the bivalve-burrow unit was lowered into running seawater. The bivalve was considered established in the plastic substrate after it had laid down byssal thread on the burrow floor and extended its siphonal process for feeding.

The course of ciliary currents was determined by introducing carborundum #100, carborundum #600, carmine, or crushed mudstone and following the movements of these substances through a Wild M-5 dissecting microscope. The light source used was a 4000 candle power fiber optics light unit manufactured by Iota Cam Company.

The corrosion-acetate technique (Fankboner, 1967), was used for casting the alimentary tract and the ducts of the digestive diverticula.

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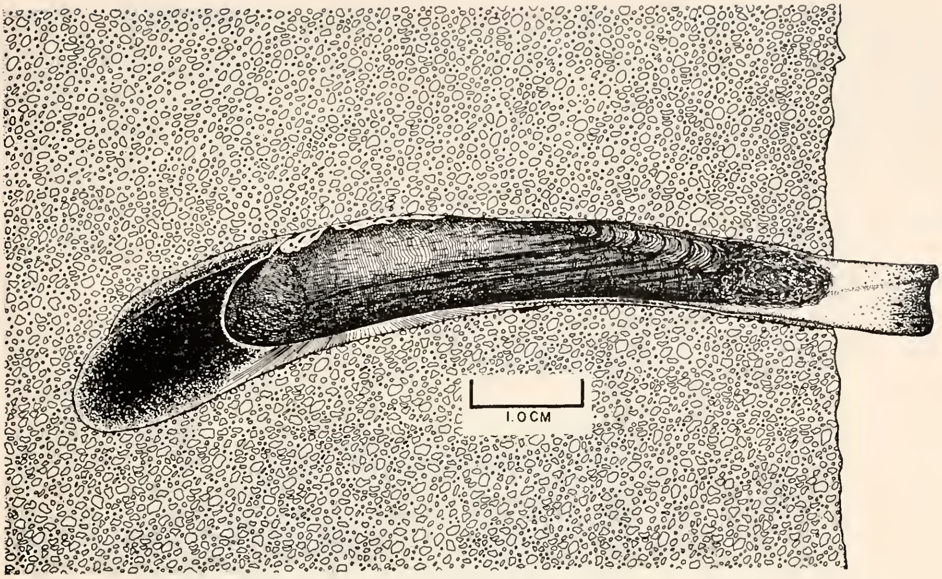

FIGURE 1. A lateral view of the burrow of *A. falcata.* The mudstone burrow has been cracked open to expose the left side of the enclosed bivalve.

RESULTS

The ciliary currents of feeding

The mantle cavity and its organs are illustrated in Figure 2. *A. falcata* feeds in the usual bivalve manner by extending its siphonal process and filtering particle-laden water through its ctenidia. Of particular interest in the mantle cavity is a flap of tissue, the siphonal valve, extending ventrally from the anterior

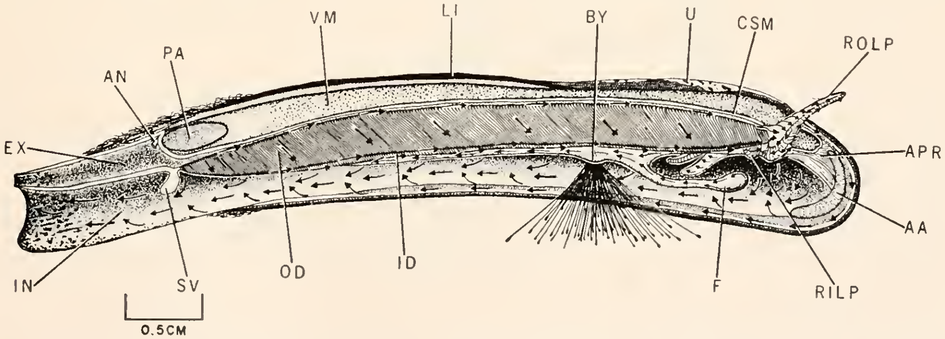

FIGURE 2. The organs and ciliary currents of the mantle cavity of *A. falcata* after removal of the right shell valve, the right mantle lobe, and part of the musculature. Abbreviations used are: AA, anterior adductor; AN, anus; APR, anterior pedal retractor; BY, byssus, CSM, cut surface of mantle lobe; EX, exhalant siphon; F, foot; ID, inner demibranch; IN, inhalant siphon; LI, ligament; OD, outer demibranch; PA, posterior adductor; RILP, right inner labial palp; ROLP, right outer labial palp; SV, siphonal valve; VM, visceral mass; U, umbone.

portion of the tissue septum separating the exhalant and inhalant siphons. The probable function of this structure will be discussed later.

The ctenidia are heterorhabdic (possessing both principal and ordinary filaments) and eleutherorhabdic (filaments not united by organic interfiliamenter junctions). However, the filaments maintain a unit integrity as they are bound together in a uniform demibranch by the intermeshed cilia of the ciliary discs (Fig. 3).

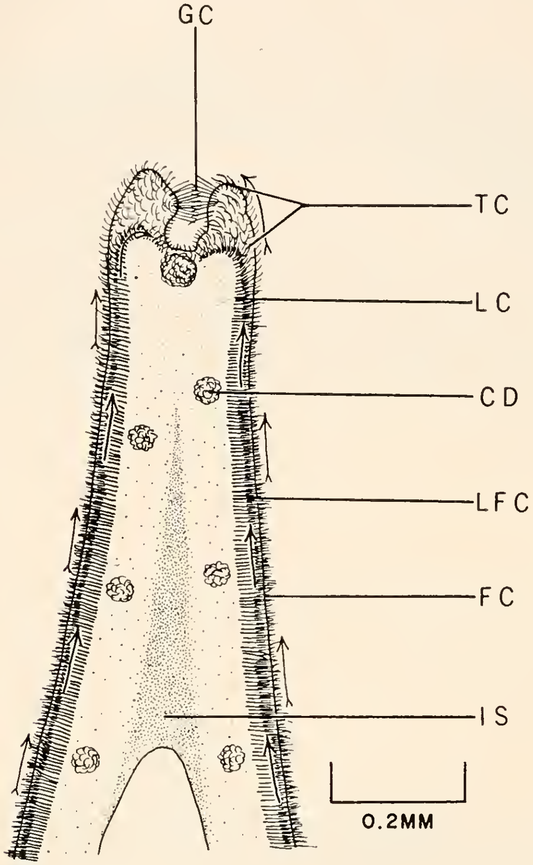

FIGURE 3. A frontal view of the ventral portion of a ctenidial filament of *A. falcata* and its ciliary currents. The feathered arrows indicate the path followed by large particles or masses of mucus-bound material. The unfeathered arrows indicate the course taken by the finer, lighter particles. Abbreviations used are: CD, ciliary disc; FC, frontal cilia; GC, guard cilia; IS, interlamellar septum; LC, lateral cilia; LFC, lateral-frontal cilia; TC, terminal cilia.

Each gill consists of two long, slender demibranchs. The inner is complete and lies slightly deeper than the anteriorly reduced outer demibranch (Figs. 2 and 3). The outer demibranchs are approximately ten filaments shorter at their anterior ends than the inner demibranchs. This condition, within the Family

Mytilidae, is not unique to *A. falcata*. Similar reductions of the outer demibranch are found in *Adula californiensis, Lithophaga bisulcata, Musculus* (*Modiolus*) *senhousei*, and *Mytilus edulis* (personal observation). Dr. Charles R. Stasek (personal communication) of Florida State University at Tallahassee, has observed the same condition in *Lithophaga plumula, Musculus* (*Modiolaria*) *laevigata, Mytilus californianus*, and *Septifer bifurcatus*. A functional advantage for this anatomical reduction is unclear but, for *Petricola pholadiformis* (a rock boring eulamellibranch with a reduced outer demibranch), much of the inner demibranch is exposed to the action of the sorting areas of the outer labial palp (Purchon, 1955a).

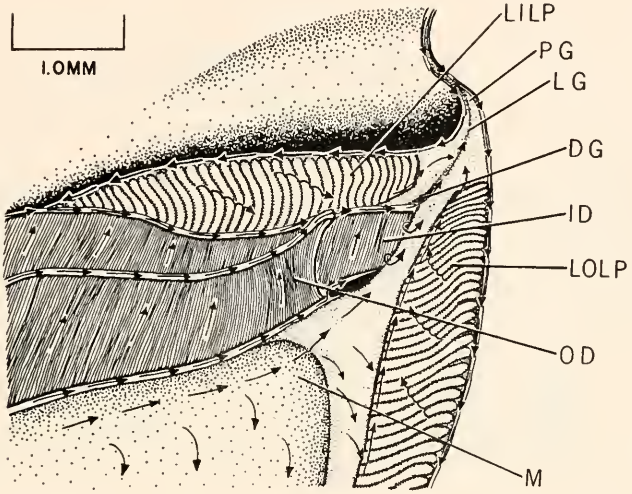

FIGURE 4. The ventral aspect of the ctenidia-palp association in *A. falcata*. Abbreviations used are: DG, distal oral groove; ID, inner demibranch; LG, lateral oral groove; LILP, left inner labial palp; LOLP, left outer labial palp; M, mantle; OD, outer demibranch; PG, proximal oral groove.

The most anterior filament on the outer demibranch is unusually large and its ventral tip has grown inward so that it conjoins its terminal food groove with that of the inner demibranch (Fig. 4). During feeding, this modified filament passes food strings to the food groove of the inner demibranch which in turn conveys them to the palps for further sorting.

The frontal ciliary currents and food grooves of the gills are identical to those of Atkin's (1937b) category B(1) for the Family Mytilidae.

The first sites of particle sorting on the gills are the adjacent tracts of fine and coarse frontal cilia (Fig. 3). The fine frontal cilia select the smaller, less dense particles and convey this material ventrally to the food grooves (Fig. 4). Once in the food grooves, these particles are passed anteriorly to the distal oral groove from where they are later conveyed by cilia to the mouth and ingested. The longer, coarse frontal cilia carry the larger particles and mucus-bound masses ventrally to the tips of the filaments, where they are passed by the terminal cilia (Fig. 3) anteriorly to the palps for further sorting (Figs. 2 and 4).

The total sorting efficiency of the outer ascending portions of the ctenidial demibranchs is associated with those areas of the mantle wall with which the gills normally come in contact. The ciliary currents of the mantle are essentially rejectory and, because they are directed ventro-posteriorly, these currents pass at right angles to the ventro-anteriorly directed currents of the ascending portions of the gill's outer demibranchs (Fig. 2). This system of ciliary currents produces a ventrally directed resultant vector which insures that large mucus-bound masses are rejected as pseudofaeces faster and with more efficiency than by means of the demibranch or the mantle wall alone.

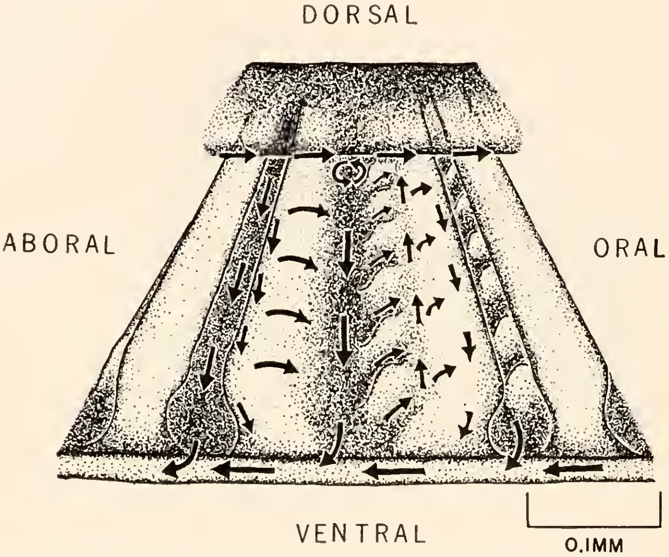

FIGURE 5. A section of the right inner labial palp of *A. falcata*, showing the palp folds and their ciliary currents.

After food is collected by the gill's cilia it is kept segregated from unsorted and/or sediment material by guard cilia projecting from the ventrolateral walls of the food grooves (Fig. 3). This separation facilitates food particles proceeding uninterrupted to the folds of the labial palps for further sorting.

In gross morphology, the paired labial palps of *A. falcata* are similar to those described by Kellogg (1915), for *M. edulis* (Figs. 2 and 4). The ciliary currents on the folds of the palps (Fig. 5) are as follows: (1) An orally-directed acceptance current passing over the crests of the palp folds; (2) A ventrally-directed rejection current moving down the crests of the palp folds; (3) An orally-directed acceptance current passing down the proximal slopes of the folds; (4) A ventrally-directed rejection current on the floor of the intrapalp fold groove; (5) An orally-directed acceptance current moving up the distal slope of the fold; (6) A dorsally directed resorting current on the upper portion of the distal slope of the fold; (7) A spiralling resorting current between the folds where they insert into the thick portion of the labial palp. The last mentioned current is probably an accumulative effect of fold currents three and five.

Particles which are accepted by the palps are carried orally over the crests of the palp folds and on to the mouth via the proximal oral groove (Fig. 4). Generally, rejected particles are passed direrctly to the ventral borders of the palps where they are collected by cilia lining the inner surface of the mantle lobes or the visceral mass (Figs. 2, 4, and 5). Subsequently, this rejected material is carried posteriorly to the embayment of the inhalant siphon (Fig. 6), and extruded from the burrow as pseudofaeces. On occasion, *A. falcata* draws the palps across the lips of its mouth in a wiping fashion, leaving particle-laden mucus-strings within the proximal oral groove (Fig. 4). This material is ingested by the mouth and conveyed to the stomach, where it undergoes further sorting and digestion.

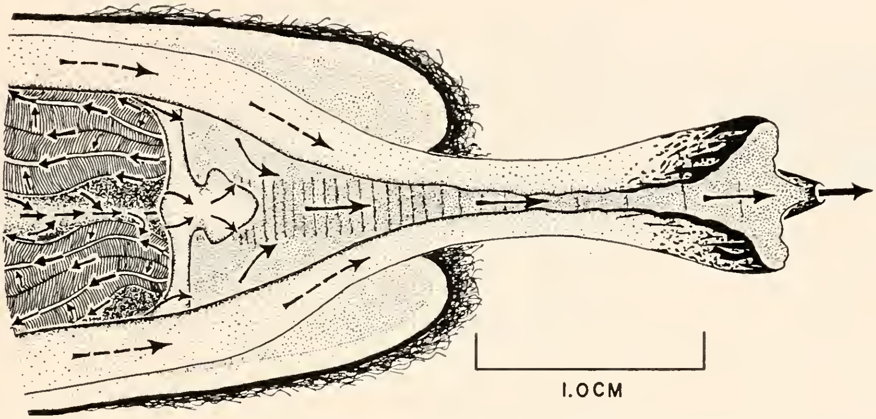

FIGURE 6. The ventral aspect of the posterior portion of the mantle cavity in *A. falcata*. The valves have been expanded to demonstrate the ciliary currents (solid arrows) of the ctenidia and the inhalant siphon. The dashed arrows indicate the direction of the currents on the inside surface of the mantle-siphon lobes.

The primary function of the labial palps of *A. falcata* is that of a sorting mechanism, but of almost equal importance is their role as a metering device, controlling the maximum volume of particles and mucus strings reaching the mouth. When the volume of food matter in the oral groove exceeds the ingestion rate of the mouth, mucus strings back up within the groove and are swept away by the rejectory cilia on the palp borders (Fig. 4). This rejected material either leaves the mantle cavity as pseudofaeces or, more rarely, is accepted by the cilia at the base of the inhalant siphon and returned to the ctenidia for resorting (Fig. 6).

The ciliary currents of digestion

The gut, associated organs, and ciliary currents of *A. falcata* are similar to those of *Botula cinnamomea* (Dinamani, 1967), *M. edulis* (Graham, 1949, and Reid, 1965), *L. gracilis* (Dinamani, 1967), *L. nasuta* (Purchon, 1957), and *Perna viridis* (Dinamani, 1967). However, because there are significant differences between *A. falcata* and these other mytilids, I will describe the stomach of

A. falcata in detail and bring out in the Discussion pertinent comparative structures and functions.

The short, ciliated oesophagus leads from the mouth to the spindle shaped mid-gut and its digestive diverticula complex (Figs. 7 and 8). The style sac terminates just anterior to the posterior adductor, while the intestine forms a flattened loop originating at the posterior stomach wall and terminating within the exhalant siphon chamber as the anus (Fig. 2).

The stomach is a complex sorting organ which houses the entrances to the caecum and the numerous openings to the dichotomies of the digestive diverticula (Fig. 7). There are sixteen duct openings to the "liver" which are equally divided into right and left duct pouches (Figs. 7 and 8). The ducts and tubules of the digestive diverticula are similar in morphology to those described by Owen (1955) for *M. edulis.* The right and left duct pouches form ciliated gutters along the floor of the stomach which veer to the left anteriorly, entering into the sorting caecum (Figs. 7 and 9). The gross ciliary currents of the pouch tracts are directed towards the caecum.

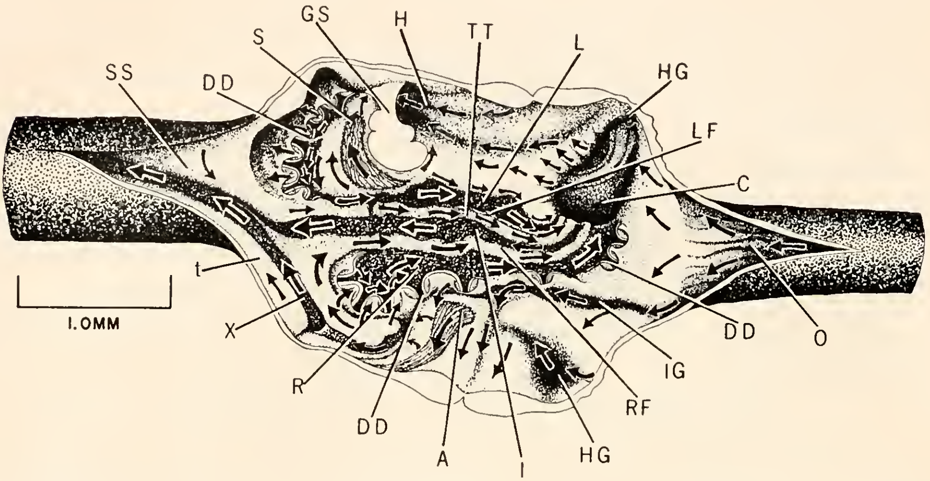

FIGURE 7. A doral view of the stomach of *A. falcata,* its structures, and its ciliary currents. Abbreviations used are: A, antero-dorsal tract; C, caecum; DD, ducts of digestive diverticula; GS, gastric shield; H, dorsal hood; HG, hood groove; I, intestinal groove; IG, minor intestinal groove; L, left duct tract; LF, left fold of duct tract; O, oesophagus; R, right duct tract; RF, right fold of duct tract; S, shield tract; SS, style sac; TT, tongue of major typhlosole; t, tongue of minor typhlosole; X, appendix.

The caecum is a short, finger-shaped pocket which opens into the stomach from the left anterior wall. The structure and the ciliary currents of this organ are illustrated in Figures 7, 8 and 9. The sorting mechanism of the caecum in *A. falcata* is similar to Reid's (1965) type B classification with one exception. At the blind end of this finger-like pocket is a structure, not found in the caecum of *M edulis,* which I call the caecal pouch (Fig. 9). This pouch envelopes the terminal end of the caecum's sorting folds and appears to physically prevent the larger particles and mucus-bound mass from entering the hood groove.

There are four dominant ciliated folds in the stomach of *A. falcata.* The shortest of these is the minor typhlosole which enters the lumen of the stomach from the style sac and extends a short distance along the right wall of the stomach (Fig. 7). The main typhlosole also originates in the style sac and continues as a meandering tongue along the floor of the stomach and disappears into the sorting caecum. Just prior to entering the caecum, it divides unequally into two folds. One of these folds is a continuation of the main typhlosole's tongue and the other becomes the left fold of the stomach (Figs. 7 and 9). The fourth fold of the stomach, the right fold, has its origin at the base of the right duct

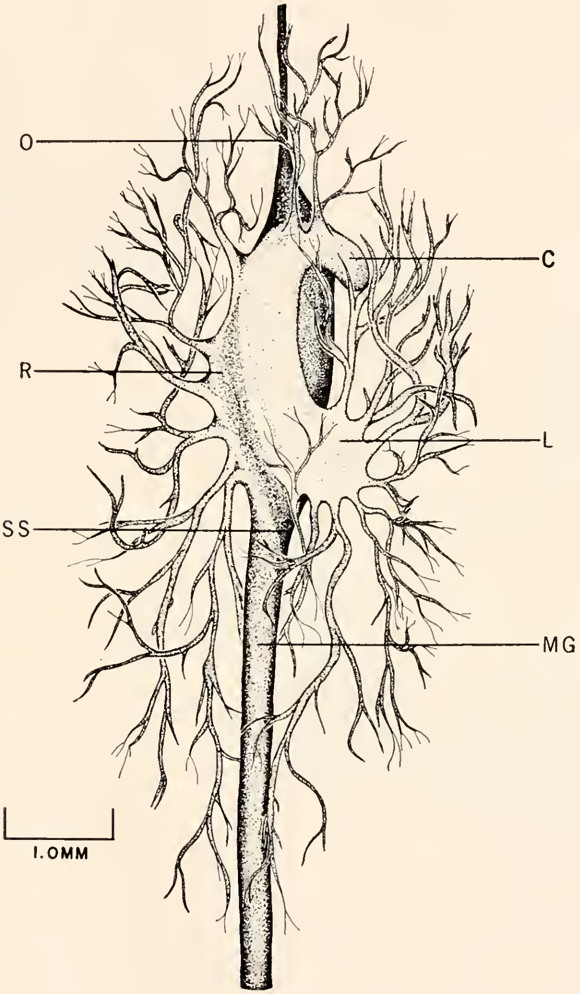

Figure 8. Drawing of the ventral aspect of a vinyl acetate cast of the alimentary tract of *A. falcata.* The duct tubules have been deleted. Abbreviations used are: C, caecum; L, left duct tract; MG, mid-gut; O, oesophagus; R, right duct tract; SS, style sac.

pouch and extends anteriorly along the floor of the stomach running parallel to the main typholosole and following it into the caecum. With the exception of the minor typholsole, all of these folds convey particles and mucus-bound materials anteriorly into the caecum for sorting.

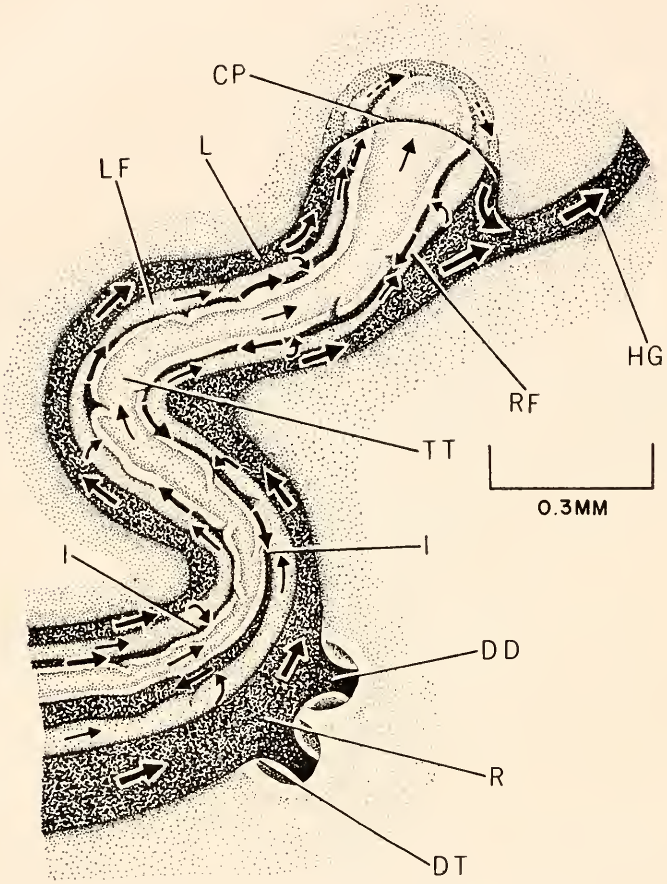

FIGURE 9. The sorting area of the caecum of *A. falcata,* its structure and ciliary currents. Abbreviations used are: CP, caecal pocket; DD, duct of the digestive diverticula; DT, duct typhlosole; HG, hood groove; I, intestinal groove; L, left duct tract; LF, left fold of duct tract; R, right duct tract; RF, right fold of duct tract; TT, tongue of major typhlosole.

The intestinal groove has its source at the point where the main typhlosole sub-divides to form the left fold of the stomach (Fig. 7), and its functions in passing materials, which have been rejected by the sorting mechanisms of the stomach, posteriorly to the mid-gut as waste material.

Forming a tributary to the intestinal groove is a crease in the right wall of the stomach, the minor intestinal groove. This groove has two functions. Its anterior half conveys particles and mucus-bound matter to ciliary currents leading to the dorsal hood; its posterior portion is rejectory in nature and passes rejected materials to the intestinal groove (Fig. 7). Just before joining the intestinal groove, the minor intestinal groove forms a shallow pocket which, in *M. edulis,* is referred to as the "appendix" by Reid (1965). The function of the appendix in *A. falcata* is to remove excess mucus-bound material from the food bolus spinning in the lumen of the stomach. When the bolus becomes too large from a high influx of food material from the oesophagus, it begins to press or extrude the excess into any grooves or pockets within the stomach wall. When such excess material is pushed into the appendix, ciliary currents convey it to the intestinal groove for expulsion from the stomach.

There are two ridged ciliated tracts in the walls of the stomach. One, the shield tract, is found on the left wall of the stomach, just posterior to the gastric shield. The second, the antero-dorsal tract, is located in a position complementary to the shield tract, on the right wall of the stomach. The ciliary currents on both tracts are relatively weak and, as far as I have been able to determine, are far less effectual than the other stomach sorting areas. *A. falcata* is a relatively advanced mytilian bivalve (Yonge, 1955) and probably its shield and anterodorsal tracts have, through time, lost the sorting activity observed by Graham (1949) and Reid (1965) in the more primitive *M. edulis.*

The translucent crystalline style projects from the style sac and its head is lodged against the lobes of the chitinous gastric shield (Fig. 7). The style's consistency is very soft and often possesses little more viscosity than a thick mucus. Occasionally, specimens of *A. falcata* have styles which contain chunks of food material embedded within the center of the rod. The presence of embedded matter appears to be related to whether the animals are freshly broken out of their mudstone burrows and examined in the field or whether they are studied after living in a marine aquarium for several weeks. In the former situation, all of the styles are clear, but those which are kept submerged and actively feeding in the laboratory tend to develop dark corkscrew spirals of chunky food material down the style centers.

The course of digestion begins following ingestion by the mouth of a particle-laden mucus string, which is moved by cilia down the oesophagus and into the stomach. Upon entering the stomach, there are several paths the mucus food string may take. The first of these paths, probably the most frequently used, is similar to that described by Reid (1965) for *M. edulis.* The food string is conveyed down the anterior wall of the stomach and is carried in an oblique direction over the right stomach fold, the main typholosole, and the left fold. It then passes posteriorly over the left wall of the stomach until it is picked up by the mucus-bound mass enclosing the head of the crystalline style (Fig. 7). The style, which, when viewed from the anterior of the animal, is turning slowly in a clockwise direction, begins to dissolve and forms a corkscrew in and around the food-mucus mass. During the spinning of the crystalline style which is coated with a "bolus" of potential food matter, small portions peel free from the turning mucus-bound mass. This is probably due to what Owen (1967) suggests is the effect of gastric fluids and abrasion by the stomach walls. Most of these food-mucus pieces drop into the right and left duct pouches, where they are either drawn into the main ducts of the digestive diverticula (Fig. 7) or are swept anteriorly into the pouch ciliary tracts and folds for later sorting in the caecum (Fig. 9).

Food entering the stomach at a rate faster than it can be sorted and utilized is bypassed to the mid-gut via the intestinal groove. When the mucus-food strings around the head of the crystalline style accumulate to excess, the material is usually scraped off into the appendix, where it is later passed to the intestinal groove and carried into the mid-gut as waste.

In the duct pouches, fine particles are taken into the main ducts of the digestive diverticula by what Owen (1955) suggests in *M. edulis* is an inhalant counter current in the nonciliated portion of the main ducts, created by an exhalant current in the ciliated portion. Owen submits that in *M. edulis* the ciliary currents of the main ducts convey waste products from the diverticula to the stomach for removal. He proposes that the counter current functions in drawing food particles towards the diverticula, where they are later digested intracellularly. *A. falcata* probably functions in a similar manner.

Particles which are passed anteriorly in the pouch tracts or the folds lying on the floor of the stomach, undergo minor sorting before they enter the caecum. The smaller, more dense particles are swept off the main typhlosole into the major intestinal groove to be later passed into the mid-gut as waste material. The larger mucus-bound masses or strings of material and the smaller, lighter particles are conveyed by the main typhlosole, the pouch folds, and, in part, by the lower left wall of the stomach, into the caecum. Once in the caecum, potential food particles are sorted by the right and left folds and the main typhlosole (Fig. 9). The heaviest finest particles are rejected into the intestinal groove, while the lighter particles are carried into the caecal pocket where cilia sweep them into the hood groove. Particles which enter the hood groove are conveyed out of the caecum and to the dorsal hood. At the head of the dorsal hood, particles and mucus-bound masses accumulate until they spill over the gastric shield and are picked up by the head of the crystalline style. The larger food strings and mucus masses either remain in the caecum, where they are eventually broken down into finer sizes, or are passed out of the caecum via the ciliated surface of the caecum's posterior wall. This material is soon caught up in the currents of the stomach's left wall and carried to the head of the dorsal hood where it is wound around the head of the style.

A second path a mucus-food string may take after entering the stomach lumen is to pass along the left anterior stomach wall into the sorting caecum. The subsequent treatment of the mucus food strings within the caecum is similar to that described in previous paragraphs.

The final alternative route which may be taken by mucus-food strings entering the stomach is to be conveyed directly from the oesophagus to the minor intestinal groove. Within this groove, ciliary currents carry the food strings posteriorly where they enter the dorsal hood and are passed to the spinning mucus-food mass or bolus enveloping the anterior portion of the crystalline style. More rarely, food strings continue in a posterior direction along the minor intestinal groove and ultimately empty into the mid-gut. On occasions when the stomach is relatively empty, food strings fall into the right duct tract and pass on to the caecum for sorting.

General comments on criteria for acceptance or rejection of particulate material by ciliary sorting mechanisms in A. falcata

Particle selection or rejection that takes place in *A. falcata,* whether by the gills, palps or stomach, appears to be based upon the size, weight, concentration, and mucus-bound state of potential food material. In general, material small in size, light in weight, in low concentration, and free of mucus is selected over larger, heavier, highly concentrated, mucus-bound particles. I observed no evidence of a selective process other than a physical one.

Sediment removal from the burrow

The fate of sediment matter entering the mantle cavity during feeding has been, in part, previously discussed. Removal of the products of mechanical boring from the burrow is initially a different process, involving active participation by portions of the mantle folds and the foot.

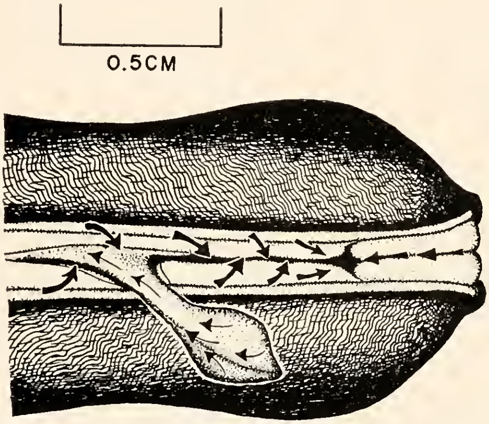

FIGURE 10. The ventral aspect of the anterior portion of *A. falcata* showing the folds of the mantle, the foot, and their ciliary currents.

Yonge (1955) suggests that in *A. falcata* sediment material resulting from mechanical boring is removed from the burrow via the folds of the mantle and the inhalant siphon. My observations on a specimen living in a clear plastic burrow confirm this. Carborundum powder (#100 and #600) which I placed on the umbones of the specimen in the plastic burrow were deposited within a few minutes at the head of the burrow. Next, the deposited particles were contacted by the anterior, fused, inner folds of the mantle margin, conveyed by cilia ventrally (with respect to the bivalve) and passed into the lumen of the mantle cavity (Figs. 2 and 10). If the carborundum particles entering the mantle cavity were introduced in large quantities, they generally became embedded in the mucus produced by the mantle epithelium and were conveyed as stringy masses posteriorly by the cilia lining the walls of the mantle cavity. On reaching the tip the inhalant siphon, the mucus-bound particles were swept away by the currents of the exhalant siphon (Fig. 6). There is one obvious advantage to pseudofaeces leaving a bivalve in a mucus-bound state. Wave action on the reef probably carries the mucus embedded material a considerable distance from the burrow opening before it breaks up. Hence, it is unlikely that the material would be introduced again in the feeding process.

When small quantities of carborundum particles were taken into the mantle cavity, they were usually conveyed to the sorting folds of the labial palps where they were treated like incoming potential food material. Finer suspended particles in the burrow are drawn into the mantle cavity through an iris-like opening formed by the anterior unfused portion of the ventral mantle marginal folds (Fig. 10). The water currents which draw in this suspended material are probably created by the slight opening and closing of the shell valves because the fluids were taken into the mantle cavity in gasping draughts rather than the steady flow characteristic of ctenidial currents. After suspended material has entered the mantle cavity, it is collected by the filtering mechanism of the gills and processed as potential food material.

The foot is long, slender, and serpentine in its movements and shape. Yonge (1955, page 387) submits: "Once the animal (*A. falcata*) is established in its boring the only, but all-important, function of the foot is to plant the byssus threads." My observations have revealed that the foot (Fig. 3) has the significant additional function of removing sediment from the walls of its mudstone burrow.

The ciliary currents of the foot are shown in Figures 2 and 10. The extensible powers of the foot are unusual. I have, on occasion, observed it expanded to at least four-fifths the length of the shell valves. There does not appear to be any portion of the walls of the mudstone burrow or the shell valves which are inaccessible to the ciliated tip of the foot. Observations on *A. falcata* in the clear plastic burrow revealed that the foot occasionally extended and touched portions of the burrow walls in a browsing manner. Further, wherever the ciliated foot touched areas of the burrow walls which were coated with #600 mesh carborundum powder, the particles were removed and passed to the mantle cavity by the foot's ciliated surfaces. Mudstone particles and carmine were also introduced and were treated in a similar manner to carborundum according to particle size, particle density, and mucus-bound state. I was never able to place more particulate material within the plastic burrow than could be handled by the mantle cavity's mechanisms for sediment removal. I was particularly impressed with the ease with which the ciliary currents of the inhalant siphon could move large, dense, mucus-bound masses (sometimes 0.5 cm in diameter) of #100 mesh carborundum powder in a vertical direction. In general, large particles were removed from the burrow as pseudofaeces via the inhalant siphon and smaller, suspended material entered the alimentary system and was later incorporated into faecal pellets.

Discussion

In particle-laden waters, many eulamellibranchs rid their mantle cavities of clogging particles by first concentrating the undesirable material into mucus-bound masses at the base of the inhalant siphon and then directing the siphonal valves so that the incurrent stream of water washes the pseudofaeces from the mantle cavity ventrally through a gape or aperture between the mantle folds (Kellogg, 1915, and Yonge, 1948). Obviously a rock boring form such as *A. falcata* cannot utilize this mechanism because it would result in effectively "fouling one's own nest." Instead, most waste material must be removed from the mantle cavity via ciliary currents of the inhalant siphon. The boring bivalves *Hiatella* and *Barnea* have a slight variation on the theme for pseudofaecal cleansing. In *Hiatella,* pseudofaeces are first consolidated into tiny balls by ciliary vortices or swirls at the base of the inhalant siphon and then later washed out through the inhalant siphon by spasmodic contractions of the clam's two adductor muscles (Hunter, 1949). The genus *Barnea* has evolved a filmy flap, the collecting membrane, which extends from the posteroventral portion of the visceral mass deep into the inhalant siphonal embayment (Kellogg, 1915, and Purchon, 1955b). Posteriorly directed ciliary currents of the collecting membrane deposit pseudofaeces into the incurrent siphon, where it is removed from the bivalve via extrusion through the siphon's opening. Such a system bypasses the problem of the stream of incoming water washing away the pseudofaeces because it effectively shields the material until it is deposited near the point of extrusion. Yonge (1955) suggests that the siphonal valve (Fig. 2 and 6) of *A. falcata* acts as a shield to protect pseudofaeces from the broad incoming stream of water from the inhalant siphon. I question this interpretation for two reasons. First, any material passing posteriorly through the inhalant siphon would obviously be subjected to the full force of the incoming water currents when it was carried by cilia over the siphonal membrane or the inner walls of the anterior portion of the mantle folds (Fig. 6). Second, *A. falcata* utilizes a gape in the ventral mantle folds just anterior to the siphonal process as a functional inhalant siphon during the removal of moderate to heavy loads of pseudofaeces. While this "functional" inhalant siphon is operative, the siphonal valve appears to work as a plug for the true siphon.

Guard cilia generally occur in those bivalves which inhabit a substrate containing some muddy or silty material (Atkins, 1937a). The mudstone burrow of *A. falcata* provides a similar habitat because sediment material from both mechanical boring within the burrow, and suspended silt from mudstone weathering outside the burrow, are deposited into the excavation. The deep marginal grooves and their guard cilia provide a mechanism for insuring that potential food material travels the full distance to the labial palps, while at the same time remaining segregated from the silt laden stream of water entering through the inhalant siphon.

Stasek (1963), has determined, from fifty-five bivalve families, that a well-defined association is present between the ctenidium and the labial palps. Based on anatomical characters, he has segregated the members of these families into three major categories. As a mytilid, *A. falcata* falls into the most primitive of these groups, Category I.

Members of Category I are characterized by the following anatomical features: ". . . the ventral tips of at least the first few or, usually, of many filaments of the inner demibranch are inserted *unfused* into a distal oral groove (a designation originated by Kellogg, 1915)" (Stasek, 1963, page 91). In *A. falcata,* and indeed in the six mytilian genera I have examined, the first few filaments of the inner demibranch are always inserted unfused into a *palp pocket* lying within the lateral oral groove (LG) (Fig. 4). Kellogg's (1915, page 629) definition of the distal oral groove states (the italics are mine): "The third is a groove in the *mantle wall,* close to and parallel with, the anterior edge of the inner demibranch, found in forms in which the outer demibranch does not extend so far forward as the inner—the distal oral groove." Kellogg further submits that there is no distal oral groove in *Mytilus,* and he is correct on the basis of his purely anatomical definition. However, Stasek (personal communication) judges that, in the Mytilidae, a functional basis for a ctenidium-palp association is more valid than a purely anatomical one. While I agree with Stasek on this point, I think that his definition for Category 1 would be more convincing if it read as follows: ". . . the ventral tips of at least the first few or, usually, of many filaments of the inner demibranch are inserted unfused into an anatomical or functional distal oral groove."

The stomach of *A. falcata* is well equipped to handle the fine sediment particles accompanying incoming food material. It differs from other mytilian rock borers, such as *B. cinnamomea* and *L. gracilis* (Dinamani, 1967), in that it possesses a well-developed sorting caecum. The folds and typhlosolar tongue of the caecum of *A. falcata* comprise the chief sorting mechanism of the stomach, as the antero-dorsal and shield tracts appear to be ineffectual. Sediment particles that get by the sorting mechanisms of the ctenidia and labial palps and are conveyed to the stomach of *A. falcata* are generally dealt with by the caecum and dumped into the intestinal groove to be carried out of the region of the stomach. The absence of a well-developed caecum in *L. gracilis* might be related to the chemical nature of its boring. *Lithophaga* spp. are usually found burrowed in a limestone substrate (Yonge, 1955) and during the boring process do not produce a particulate sediment. Hence, they probably do not require the sorting mechanism found in the stomach caecum of *A. falcata.*

Dinamani (1967) has suggested that in the Mytilidae the caecum functions more as a temporary reservoir of food than as a sorting mechanism. He observed that, in specimens of *P. viridis* starved for up to two days, the food matter within the caecum had disappeared. The caecum may have a purely storage function in mytilids where the sorting folds of the caecum are rudimentary, such as in *B. cinnamomea, L. gracilis,* and *P. viridis,* but in *A. falcata* and *M. edulis,* where the sorting mechanism is well-developed, the caecum is patently a device for resolving food matter from mucus and sediment particles.

The complexity and functional significance of the appendix in the bivalve stomach ranges from a rudimentary, static groove in *M. edulis* (Reid, 1965) to the large food storage pouch found in the wood-boring Teredinidae (Purchon, 1968). Yonge (1949) submits that the appendix or antero-dorsal caecum in tellinids functions as both a storage cavity for sand grains to aid in trituration of food material, and a relief valve for excess food-mucus strings accumulating around the head of the crystalline style. The rejectory currents of the appendix of *A. falcata* precludes its functioning in a storage capacity. Moreover, I was never able to observe the overt abstraction of mudstone particles in any portion of the stomach. The appendix of *A. falcata* functions solely as a relief mechanism, and, instead of temporarily storing the bolus material pressed into it, the ciliary currents of the appendix convey this material to the intestinal groove, where it is subsequently removed from the stomach. The obvious advantage of the appendix to *A. falcata* is that the presence of a relief mechanism in the stomach prevents overeating and thus maintains the proper volume for optimum mixing of food with gastric juices.

The crystalline style from specimens of *A. falcata* maintained in aquaria often contained bits and pieces of food matter. Morton (1952) suggests that this is a common occurrence which is advantageous to the style bearer in that it permits recovery and digestion of food matter which would otherwise be lost as faeces. Nelson (1918) found similar structures in the styles of *Anodonta grandis* and *Modiolus modiolus.* Nelson's explanation for this phenomenon was that, during the formation of the crystalline style, a clear mucus stream is secreted by the walls of the intestine and picked up by the ciliated surfaces of the typhlosoles in the mid-gut. During the process of the mucus stream's being carried to the typhlosoles, it is twisted on itself and forms a corkscrew spiral. Frequently, odd particles of food are picked up by the twisted mucus stream and this accounts for the dark color of the spiral structure. Nelson further suggests that if an animal is starved, loses its style, and then suddenly starts feeding again, it usually generates a dark cored new style. In the case of *A. falcata,* continuous feeding with loss and subsequent regeneration of the style, as is probably the case in the subtidal zone as well as in aquaria, is the more common cause for the dark cored style being produced. Doubtless, as Morton (1952) has suggested, it is advantageous to the style bearer to recycle food material which would otherwise be lost, but on the other hand, the amounts of food involved in this process are probably too minute to be of any real importance to nutrition in the majority of the Bivalvia.

Functionally, the crystalline style supplies most, but not all, of the digestive enzymes found in the lumen of the stomachs of style-bearing bivalves (Reid, 1968). According to Morton (1967), the style acts as a stirring rod and windlass as well. I found no evidence of the crystalline style of *A. falcata* functioning as a windlass for the string of mucus-bound food emerging from the posterior end of the oesophagus. The physics of Morton's observation suggest that there would have to exist some mechanism for increasing the rate of the food string moving down the oesophagus as the bolus of material around the style increased in diameter. Otherwise, as the first wrappings were going on the style, there would be a windlass mechanism, but as the bolus diameter increased, so would the rate of wrapping increase (if one can assume that the rate of the rod's turning is constant) and the food string would break from the strain. Another possibility is that, as the food bolus increases in diameter, increased friction with the walls of the stomach would act as a clutch, slowing the turning rate of the style and, thus, keeping the wrapping speed of food strings uniform.

My observations on conditions associated with the acceptance or rejection of particulate material in *A. falcata* suggest that its sorting mechanisms are not highly efficient. However, permitting the passage of some sediment material into the stomach could be advantageous to the animal's well-being. The presence of sediment matter in the gut may aid in trituration of food, as is the case in tellinids (Yonge, 1949). More likely, the consolidation of sediment particles into firm faecal pellets prevents reintroduction of that material into the mantle cavity or burrow. Reid and Reid (1969) report a sand-grain epiflora in the stomach of *Macoma secta* which comprises most of its nutrients. Since, volume for volume, fine sediment particles would possess a surface area proportionally higher than that of sand grains, it is possible that the small amount of sediment material in the stomach of *A. falcata* provides nutritional value as well.

Adaptative radiation of the bivalve foot is illustrated most commonly in the function of locomotion. The creeping of the saddle oyster *Enigmonia aenigmatica* (Yonge, 1957), *Lasaea rubra* climbing a rock face via adhesion to a slime trail (Morton, 1960), and the leaping and digging-in of cockles are all executed by the bivalve's use of the foot. Within rock boring bivalves, if the foot is functional at all, it is utilized for stabilization of the visceral mass during boring, either by the laying-down of a byssus in the case of *Adula, Lithophaga,* and *Tridacna* (Yonge, 1955 and 1936) or in functioning as a sucker, gripping the head of the burrow, as reported by Purchon (1955b) for the Pholadidae. In *A. falcata,* the foot performs the additional function of cleansing its burrow walls of the particulate mudstone produced during mechanical boring.

I wish to express my gratitude to Dr. Charles R. Stasek, formerly of the California Academy of Sciences, for his advice and encouragement during the course of this study. Thanks are also due to Dr. E. H. Smith, Director of the Pacific Marine Station, for providing the facilities where much of this work was completed, and to Professor A. R. Fontaine, Professor G. O. Mackie, Dr. R. G. B. Reid, and Dr. R. A. Ring, of the University of Victoria, for comments and criticism of the manuscript. Excess figure charges were paid from a NRC operating grant A-1427 to Dr. G. O. Mackie, Victoria, Canada.

SUMMARY

The mode of sediment removal by *A. falcata,* and its relationship to the ciliary currents of feeding and digestion was determined. Where possible, morphological and functional comparisons were made with other rock-boring bivalves.

1. *A. falcata* moves sediment material from the walls of its burrow into its mantle cavity by means of the ciliated surfaces of its unusually extensible foot and the mantle folds. After entering the bivalve's mantle cavity, sediment particles are treated as potential food material by the ciliary sorting mechanisms associated with feeding and digestion. Most sediment matter is rejected from the burrow as pseudofaeces via extrusion through the inhalant siphon opening. However, small amounts of sediment particles find their way past rejectory mechanisms of the gills and labial palps, and pass into the stomach. Following sorting and subsequent rejection by the stomach, this material is incorporated into faecal pellets which are later swept from the area of the burrow by the strong currents of the exhalant siphon.

2. It is suggested that particle discrimination, during sorting by *A. falcata,* is dictated by the weight, size, and mucus-bound state of the material being sorted.

LITERATURE CITED

ATKINS, D., 1937a. On the ciliary mechanisms and inter-relationships of lamellibranchs. Part II: sorting devices on the gills. *Quart. J. Microscop. Sci.* **79**: 339–373.

ATKINS, D., 1937b. On the ciliary mechanisms and inter-relationships of lamellibranchs. Part III: types of lamellibranch gills and their food currents. *Quart. J. Microscop. Sci.,* **79**: 375–421.

DINAMANI, P., 1967. Variation in the structure of the stomach in the Bivalvia. *Malacologia,* **5**(2): 225–256.

Fankboner, P. V., 1967. The corrosion-vinyl acetate technique as an aid in the reconstruction of the marine molluscan alimentary system. *Veliger,* **9**(4): 444–446.

Graham, A., 1949. The molluscan stomach. *Trans. Roy. Soc. Edinburgh,* **61**(27): 737–778.

Hunter, W. R., 1949. The structure and behavior of *Hiatella gallicana* L. and *H. artica* L, with special reference to the boring habit. *Proc. Roy. Soc. Edinburgh,* **63B**: 271–289.

Kellogg, J. L., 1915. Ciliary mechanisms of lamellibranchs with descriptions of anatomy. *J. Morphol.,* **26**: 625–701.

Morton, J. E., 1952. The role of the crystalline style. *Proc. Malacol. Soc. London,* **29**: 85–92.

Morton, J. E., 1960. The responses and orientation of the bivalve *Lasaea rubra* Montagu. *J. Marine Biol. Ass. U.K.,* **39**: 5–25.

Morton, J. E., 1967. *Molluscs.* Hutchinson University Library, London, 244 pp.

Nelson, T. C., 1918. On the origin, nature, and function of the crystalline style of lamellibranchs. *J. Morphol.,* **31**(1): 53–111.

Owen, G., 1955. Observations on the stomach and the digestive diverticula of the Lamellibranchia. Part I. The Anisomyaria and Eulamellibranchia. *Quart. J. Microscop. Sci.,* **96**: 517–537.

Owen, G., 1967. Digestion. Pages 53–96 in K. M. Wilbur, C. M. Yonge, Eds., *Physiology of Mollusca, Volume II.* Academic Press, New York.

Purchon, R. D., 1955a. The functional morphology of the rock-boring lamellibranch *Petricola pholadiformis* L. *J. Marine Biol. Ass. U.K.,* **34**: 257–278.

Purchon, R. D., 1955b. The functional morphology of the British Pholadidae (rock-boring Lamellibranchia). *Proc. Zool. Soc. London,* **124**: 859–911.

Purchon, R. D., 1957. The stomach in the Filibranchia and Pseudo-lamellibranchia. *Proc. Zool. Soc. London,* **129**: 27–60.

Purchon, R. D., 1968. *The Biology of the Mollusca.* Pergamon Press Ltd., London, 560 pp.

Reid, R. G. B., 1965. The structure and function of the stomach in bivalved molluscs. *J. Zool.,* **147**: 156–184.

Reid, R. G. B., 1968. The distribution of digestive tract enzymes in lamellibranch bivalves. *Comp. Biochem. Physiol.,* **24**: 727–744.

Reid, R. G. B., and A. Reid, 1969. Feeding processes of members of the genus *Macoma* (Mollusca: Bivalvia). *Can. J. Zool.,* **47**: 649–657.

Stasek, C. R., 1963. Synopsis and discussion of the association of ctenidia and labial palps in the bivalved Mollusca. *Veliger,* **6**(2): 91–97.

Yonge, C. M., 1936. Mode of life, feeding, digestion and symbiosis with zooxanthellae in the Tridacnidae. *Sci. Repts. Great Barrier Reef Exped.* (*British Museum Nat. Hist.*), **1**: 283–321.

Yonge, C. M., 1948. Cleansing mechanisms and the function of the fourth pallial aperture in *Spisula subtruncata* Da Costa and *Lutraria lutraria* L. *J. Marine Biol. Ass. U.K.,* **27**: 585–596.

Yonge, C. M., 1949. On the structure and adaptations of the Tellinacea, deposit-feeding Eulamellibranchia. *Phil. Trans. Roy. Soc. London Series B,* **234**: 29–76.

Yonge, C. M., 1955. Adaptation to rock-boring in *Botula* and *Lithophaga* (Lamellibranchia, Mytilidae) with a discussion on the evolution of this habit. *Quart. J. Microscop. Sci.,* **96**: 383–410.

Yonge, C. M., 1957. *Enigmonia aenigmatica* Sowerby, a motile anomiid (saddle oyster). *Nature,* **180**: 765–766.

Reference: *Biol. Bull.*, **140**: 46–62. (February, 1971)

TRANSPORT OF SUGARS IN THE TAPEWORM *CALLIOBOTHRIUM VERTICILLATUM*[1]

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Since tapeworms have no digestive tract and no cavities in the body other than the so-called osmoregulatory canals and genital ducts, it has been assumed that nutrients enter the body through the outer surface. Further, several tapeworm species are known to require an external source of carbohydrate for growth and reproduction; at least 14 species can metabolize no sugars other than glucose and galactose (Read and Simmons, 1963). *Calliobothrium verticillatum*, a tetraphyllidean cestode parasitizing the smooth dogfish, has been reported to absorb and metabolize glucose and galactose but not to utilize fructose, mannose, sucrose, lactose, trehalose, or maltose from the suspending medium (Read, 1957; Laurie, 1961). It seems highly probable that glucose, and perhaps galactose, is a required energy source for *Calliobothrium*. Although no data on the concentrations of free monosaccharides in the environment of the worm are available, it seemed desirable to study sugar absorption by the worm *in vitro*, with the view that sugar metabolism may limit growth and reproduction of *Calliobothrium* in its host.

MATERIALS AND METHODS

The hosts, *Mustelus canis*, were collected by a commercial fisherman and distributed by the Supply Department of the Marine Biological Laboratory. The dogfish were maintained in large tanks with running sea water (18–22°) until the time of an experiment. The holding period varied from one to three days. The hosts were killed by a blow on the chondrocranium and the spiral intestine quickly removed to a container immersed in ice. The intestinal valves were split longitudinally and the adult cestodes removed to a balanced salt solution also maintained at 0° (250 m*M* NaCl, 4.4 m*M* KCl, 5.1 m*M* $CaCl_2$, 2.9 m*M* $MgCl_2$, and 300 m*M* urea, buffered with 10 m*M* tris-maleate at pH 7.2, as described by Read, Simmons, Campbell and Rothman 1960). This solution is referred to herein as saline. The parasites were freed of adhering intestinal contents and mucus and sorted into groups of about eight to ten worms. Each sample was placed in 4 ml of saline in a 30 ml beaker. Beakers containing the parasites were preincubated in a shaking water bath at 20° for 30 minutes. Unless otherwise stated, all incubations were carried out at 20° C. At the end of an incubation, worms were rapidly removed from the incubation medium, quickly rinsed three times in saline, blotted on hard filter paper, and placed in 2 ml of 70% ethanol. Extraction of soluble materials was carried out at room temperature for at least

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18 hours with frequent agitation. Incubation times and other manipulations will be discussed in the context of the experiments.

Wet weights of parasites were determined on a torsion balance. Dry weights and ethanol-extracted dry weights were obtained after drying the material to constant weight at 103° C in small tared aluminum cups. Estimation of parasite body water was made gravimetrically, *i.e.,* wet weight minus dry weight.

Isotopic methods

Radiochemicals were obtained from New England Nuclear Corporation and Cambridge Nuclear Corporation. Radioactivity was determined on aliquots of ethanol extracts or incubation media using a low background gas flow counter (C^{14}) or a gamma ray spectrometer with a NaI crystal (K^{42} and Na^{24}). Specific activities (c/m μmole^{-1} or c/m meq^{-1}) were determined by counting aliquots of various media after appropriate dilution with 70% ethanol.

Chemical methods

All unlabeled carbohydrates were purchased from commercial sources (Mann Research Laboratories, Pfanstiehl Chemical Co. and Sigma Chemical Co.).

Polysaccharide and total ethanol-soluble carbohydrate were determined by the phenol-sulfuric acid method of Dubois, Gilles, Hamilton, Rebers and Smith (1956). Polysaccharide was insolated by digestion at 100° C in 30% KOH and precipitation with 1.2 volumes of 95% ethanol. For determination of the specific activity of the polysaccharide (μmoles ^{14}C-glucose incorporated/μmole glucose in polysaccharide), the precipitated material was washed five times with 70% ethanol containing 0.1% LiCl, by alternate dispersion and centrifugation. This was found to be necessary for the complete removal of ethanol-soluble radioactivity. The washed pellet was then dissolved in water and aliquots removed for the determination of radioactivity and total carbohydrate, using glucose or trehalose standards.

Glucose was determined on aliquots of extracts or media by the glucose oxidase method ("Special Glucostat," Worthington Biochemical Corp.). The purified enzyme preparation was employed to minimize errors due to other carbohydrates in the experimental materials.

Sodium and potassium were determined by flame photometry, using a Coleman Junior spectrophotometer with a flame attachment. Standard solutions contained equivalent amounts of each ion in the chloride form and ethanol at the same concentration as samples. All inorganic reagents were obtained from Fisher Scientific Co. or J. T. Baker Chemical Co.

RESULTS

Effects of the gas phase

Since carbon dioxide is known to affect carbohydrate metabolism in a number of animal parasites (von Brand, 1966; Prichard and Schofield, 1968; McDaniel, Read and MacInnis, in press), it seemed advisable to determine whether the gas phase affected the absorption of glucose in *Calliobothrium.* Data from such experiments are shown in Figures 1 and 2. When compared with the results obtained in air, nitrogen-carbon dioxide had no significant effect on the absorption or accumulation of glucose, but had a dramatic effect on the incorporation of ^{14}C-glucose into glycogen. This effect is clearly due to the presence of carbon dioxide in the atmosphere since McDaniel *et al.* (in press) showed that under nitrogen or carbon dioxide-free air, incorporation of ^{14}C-glucose into the glycogen of *Calliobothrium* was markedly less than that observed when the gas phase contained carbon dioxide. Since absorption was not so affected, subsequent experiments were carried out in air. It may be pointed out that, under the conditions of the experiments shown in Figures 1 and 2, the kinetics of absorption were

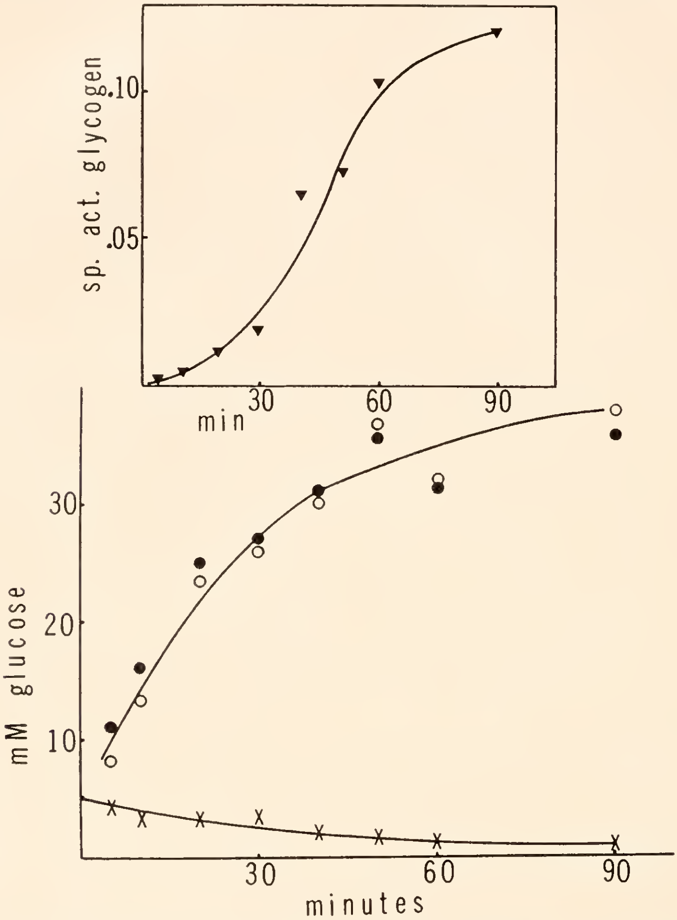

FIGURE 1. The absorption of ^{14}C-glucose by *Calliobothrium* under atmosphere of 95% N_2–5% CO_2. Bicarbonate was added to maintain pH at 7.2. Glucose in worms was determined by glucose oxidase (●) or by radioactivity (○). Glucose in the medium was determined by glucose oxidase (×). Inset's: Incorporation of ^{14}C-glucose into glycogen in same worm samples expressed as specific activity (micromoles ^{14}C-glucose per micromole of glycogen glucose). Each point in all curves is the average of worm samples. Compare with Figure 2.

first order. Further, at concentrations which probably resemble those of the worm's environment, the worm clearly accumulates glucose against a concentration difference (Table 1). Later experiments were designed to determine whether or not this was a saturable system.

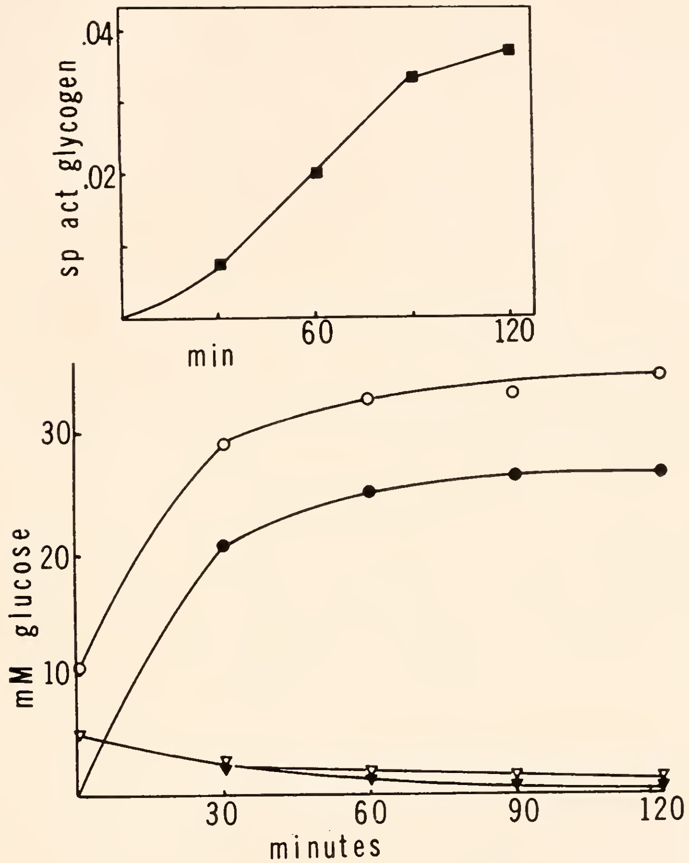

FIGURE 2. The absorption of ^{14}C-glucose by *Calliobothrium* under atmosphere of air Glucose in worms determined by glucose oxidase (○) or by radioactivity (●). Similarly, glucose in the medium was determined by glucose oxidase (▲) or by radioactivity (△). Inset is: Incorporation of ^{14}C-glucose into glycogen in same worm samples expressed as specific activity (micromoles ^{14}C-glucose per micromole of glycogen glucose). Each point in all curves is the average of two worm samples. Compare these data with Figure 1.

Effect of temperature

When worms were incubated with glucose at each of several temperatures for 60 minutes, sugar accumulation rose sharply with temperature to a maximum at about 20° C. Above 30° C, absorption sharply declined (Fig. 3). The data do not allow a very precise estimate of the optimum temperature for glucose accumulation, but the extent to which glucose is incorporated into glycogen appears to have a higher temperature optimum than that for glucose accumulation (Fig. 3).

TABLE I

Effect of time on the accumulation of glucose by Calliobothrium. *Incubation mixture contained 5 ml of 0.5 m*M *glucose in KRT. Results based on average of two determinations*

Incubation time (minutes)	Glucose conc. inside (m*M*)	Glucose conc. outside (m*M*)	$\frac{\text{Conc.}_i}{\text{Conc.}_o}$
0	4.9	0.5	9.8
1	5.3	0.46	11.5
2	6.8	0.43	15.8
4	7.7	0.38	20.3
6	8.5	0.36	23.5
8	9.0	0.29	31.0
10	10.4	0.28	37.0
20	12.1	0.12	100.8
30	12.6	0.04	315.0

Effect of pH

The absorption of glucose was affected by hydrogen ion concentration; the highest rates were observed at about pH 8 to 9 (Fig. 4). There was a dramatic

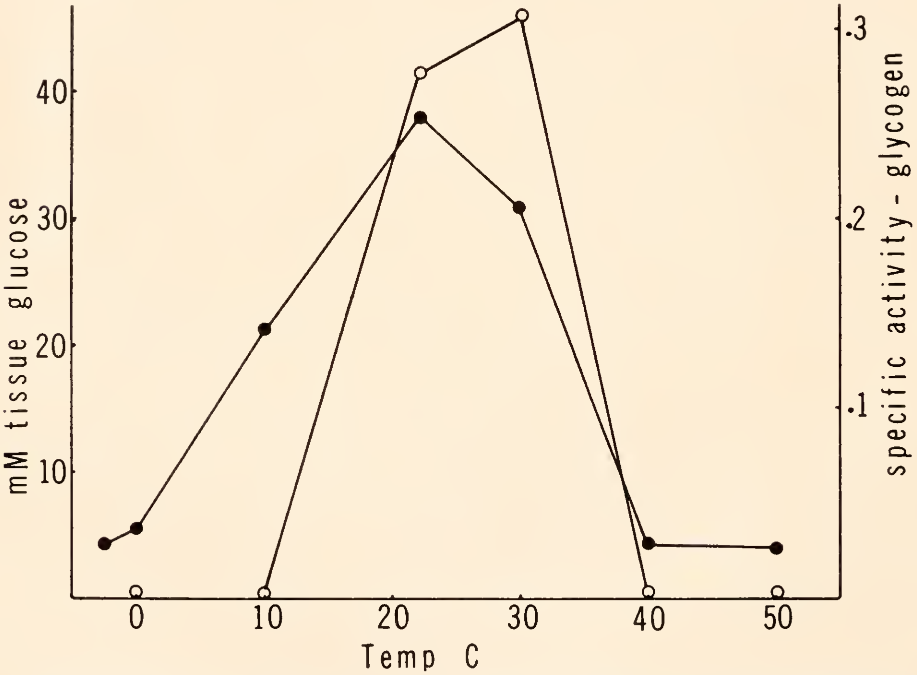

FIGURE 3. Effect of temperature on the 60 minute accumulation of tissue glucose (●) and on incorporation of ^{14}C-glucose into glycogen (○). Tissue glucose was determined by glucose oxidase. Specific activity is µmoles ^{14}C-glucose per µmole of glycogen glucose. The medium contained 5 m*M* glucose at the beginning of the experiment. Each point is the average of two samples.

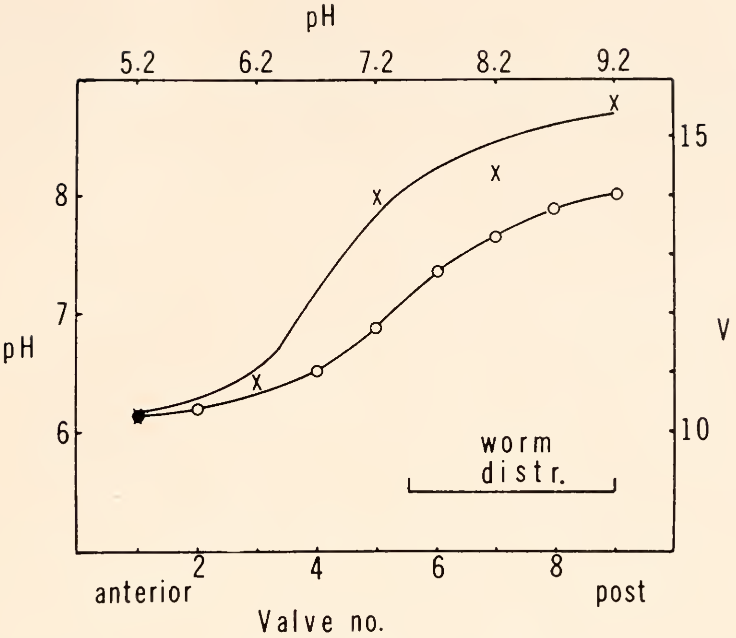

FIGURE 4. Effect of pH on rate of ^{14}C-glucose absorption in 2 min incubations (×) and the pH of intestinal contents in different segments of the dogfish spiral intestine (○). Worm samples were preincubated for 60 minutes in buffered saline at the appropriate pH and then incubated with 0.01 m*M* ^{14}C-glucose at the same pH as the preincubation medium. The V = μmoles per gram dry wt per hr. Normal worm distribution in the spiral intestine is indicated.

TABLE II

The effect of various compounds on the transport of glucose by Calliobothrium. *Inhibitor concentration, 5 m*M; *substrate concentration, 0.05 m*M: *Incubation time 2 min*

Inhibitor	% Inhib.	No inhibitory effect	
Glucose	75.6	n-acetyl-glucosamine	alanine
Arbutin	73.2	D-glucosamine	arginine
Maltose	59.9	lactose	aspartic acid
α-Methylglucoside	58.6	fructose	glycine
Galactose	44.2	mannose	leucine
Cellobiose	25.2	3-0-methylglucoside	methionine
Salicin	23.2	rhamnose	proline
Ouabain*	24.5	sorbose	taurine
Phlorizin**	96	sucrose	trimethylamine
			valine

* Present at 2 m*M*.
** Present at 0.01 m*M*.

drop in the entry kinetics between 7.2 and 6.2. Determination of the pH in various segments of the spiral intestine of *Mustelus* revealed that there is a pH gradient. In the region of the first spiral, the pH is about 6 and rises to a pH of about 8 in the ninth spiral. These data are shown in Figure 4 and the observed distribution of *Calliobothrium* strobilae in the spiral intestine is shown for comparative purposes. The worm appears to inhabit those segments of the spiral intestine having a pH range which is highly favorable for the absorption of glucose by the parasite. It would be of interest to examine the effect of pH on the absorption of glucose by free segments of *Calliobothrium,* since these are normally found in the first two spirals where the pH is relatively low. Attempts to make such determinations have proven to be technically difficult.

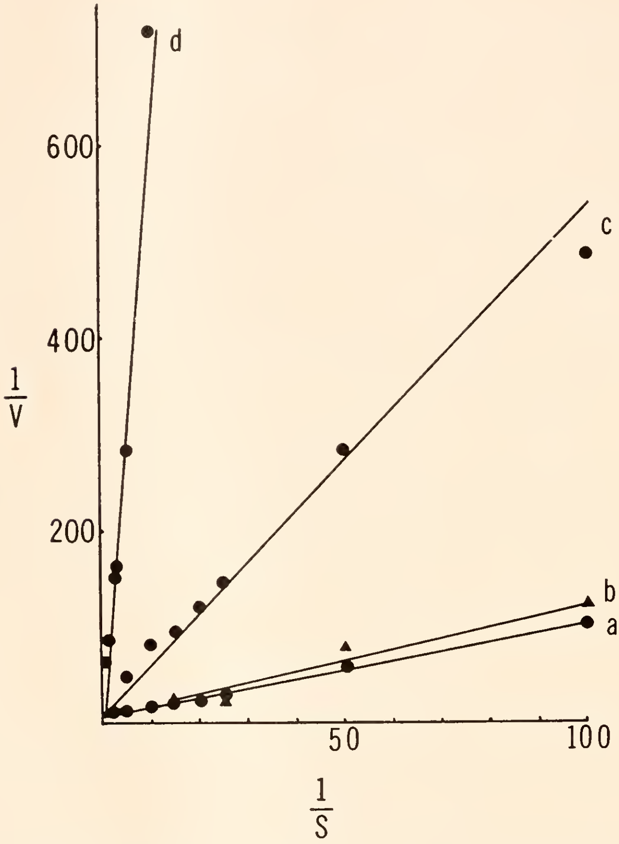

FIGURE 5. Lineweaver-Burke plots of ^{14}C-glucose absorption in 2-min incubations without inhibitor (a) or with 0.01 m*M* phloretin (b) or 0.01 m*M* phlorizin (d). Samples of curve c were incubated for 60 minutes in 0.01 m*M* phlorizin, rinsed, and incubated for 2 min with ^{14}C-glucose. Each point is average of two samples. Abbreviations are: V = μmoles per gram per hour; S = m*M* ^{14}C-glucose.

Effects of inhibitors

In 2 min incubations, 5 m*M* 2,4-dinitrophenol had no effect on the rate of glucose transport. However, when the worms were preincubated for 10 min in 5 m*M* dinitrophenol, glucose absorption was inhibited 30% in a subsequent 2 min incubation without dinitrophenol. Sodium iodoacetate at 0.5 m*M* produced a 30% inhibition of glucose transport in 2 min incubations.

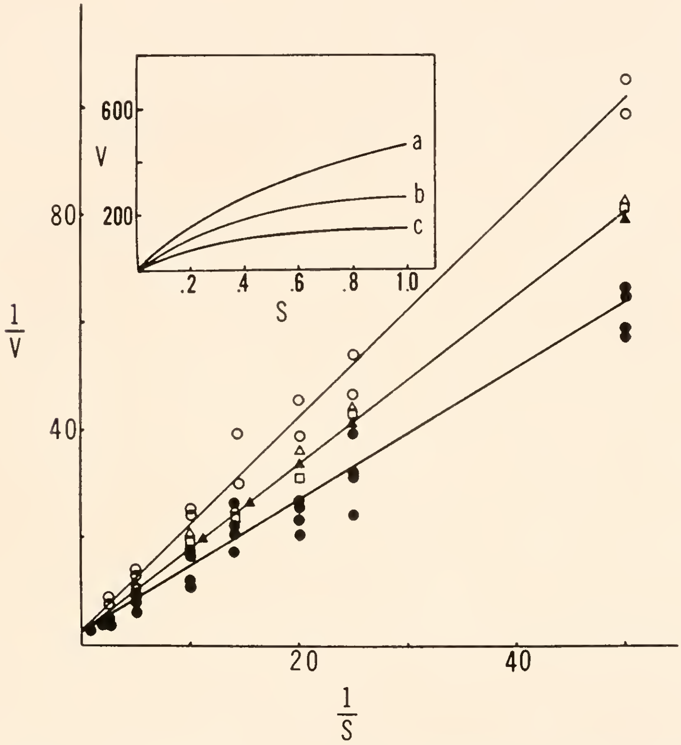

FIGURE 6. Lineweaver-Burke plots of ^{14}C-glucose absorption in 2-min incubations in the presence of galactose (△), maltose (□), α-methylglucoside (▲), ouabain (○), or without inhibitor (●). Inhibitors were present at 2 m*M*. Points for curves with galactose, maltose, and α-methylglucoside as inhibitors are averages of two determinations while those of the ouabain-inhibited and uninhibited glucose absorption curves are individual determinations. Inset is: Curves for absorption of glucose alone (a), glucose in the presence of 2 m*M* maltose (b), and glucose in the presence of 2 m*M* ouabain (c). Data points from the lower curves were used to construct inset curves. Abbreviations are: S = m*M* glucose; V = μmoles absorbed per gram dry weight per hour.

Twenty-eight additional compounds were examined as inhibitors of glucose transport (Table II). Of those tested, eight produced significant inhibitions. When these same compounds were tested as stimulators of the efflux of previously accumulated glucose, only those which inhibited glucose absorption caused an enhanced leakage of glucose. Further, sodium α-ketoglutarate, β-glycerophosphate, or dithiazanine had no effect on efflux of previously accumulated glucose.

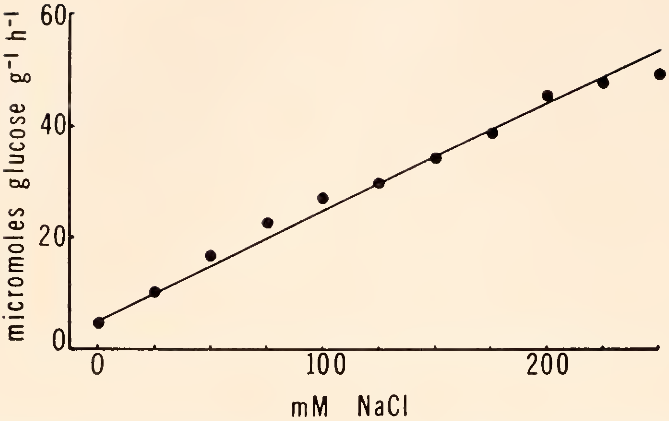

FIGURE 7. Effect of Na^+ on the absorption of ^{14}C-glucose in 2-min incubation. Deleted NaCl was replaced by KCl. Glucose was present at 0.2 m*M* in all incubates and each point is average of two samples.

The latter compound is an anthelminthic drug which blocks sugar transport in the isolated gut of *Ascaris* (Fisher, unpublished) but has no effect on glucose uptake or efflux in *Calliobothrium.*

Phlorizin, which is known to be a strong inhibitor of glucose transport in other tapeworms (Phifer, 1960; Laurie, 1961), as well as various other cells and tissues, was the most powerful inhibitor of glucose transport in *Calliobothrium.* The inhibition appeared to be competitive in character in 2 min incubations (Fig. 5). However, when worms were incubated for 60 min in the presence of 0.01 m*M* phlorizin and rinsed in phlorizin-free saline, the inhibition of glucose uptake was only partially reversed (Fig. 5c). The effect of phlorizin is apparently dependent on the intact glycoside since the aglycone, phloretin, produced a negligible inhibition (Fig. 5b).

Ouabain, a cardiac glycoside, produced a significant inhibition of glucose transport, but the glycone moiety of ouabain, rhamnose, was not inhibitory (Table II). Two other glycosides, arbutin and salicin, had significant activity as inhibitors of glucose transport (Table II).

The inhibitions produced by galactose, α-methylglucoside, maltose, and ouabain were examined in some detail. Lineweaver-Burke plots of the data indicated that the inhibitions are competitive in character (Fig. 6).

TABLE III

Effect of glucose entry on the ionic composition and wet weight of Calliobothrium

Incubation time (minutes)	K^+ meq. l^{-1}	Na^+ meq. l^{-1}	Wet weight increase (mgm%)
0	87	172	—
30	79	203	7.3
60	73	221	13.5
90	71	229	19.5
120	70	234	32.2

Na and K Effects

Since sodium has been implicated in the transport of sugars and amino acids in other animal cells (Stein, 1967), it was deemed desirable to determine whether sodium is involved in glucose transport in *Calliobothrium.* When K^+ was substituted for Na^+ in the saline, the rate of glucose transport was a linear function of Na concentration (Fig. 7). Further, it was found that tissue sodium increased when glucose was being absorbed, as might be expected in the co-transport of sodium and glucose. The increase in sodium was accompanied by less than compensatory decrease in tissue potassium and by an increase in tissue water (Table III). Control worms incubated in saline without glucose showed no significant changes in tissue sodium, potassium, or wet weight.

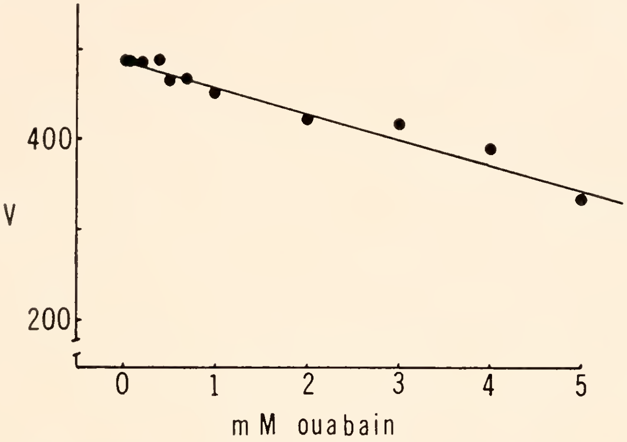

FIGURE 8. The effect of various concentrations of ouabain on the absorption of 5 m*M* ^{14}C-glucose in 2-min incubations of *Calliobothrium.* The V = μmoles absorbed per gram dry weight per hour.

As previously noted, the glycoside ouabain inhibits glucose transport in *Calliobothrium.* In 2 min incubations, the inhibition increases linearly between 0 and 5 m*M* ouabain (Fig. 8). This inhibitor is known to act on sodium transport in a number of animal cells, blocking the sodium extrusion mechanism. In *Calliobothrium,* ouabain causes a marked net influx of sodium (Fig. 9). Exposure to ouabain causes the tissue sodium to rise from about 180 to 300 meq/l in 30 minutes. This is accompanied by an enhanced net efflux of potassium which is not equivalent to sodium influx. Glucose partially alleviates the effect of ouabain on the fluxes of both sodium and potassium. It may also be noted in Figure 8 that ouabain inhibits the net accumulation of glucose by *Calliobothrium,* reducing it by about 63%.

If ouabain acts on the system involved in maintaining sodium at internal concentrations which are lower than that of the ambient medium, it might be expected to affect efflux of sodium from the worm. To test this hypothesis, worms were equilibrated for 60 min in saline containing Na^{24}, following which the worm samples were transferred repetitively at 5 min intervals to vessels

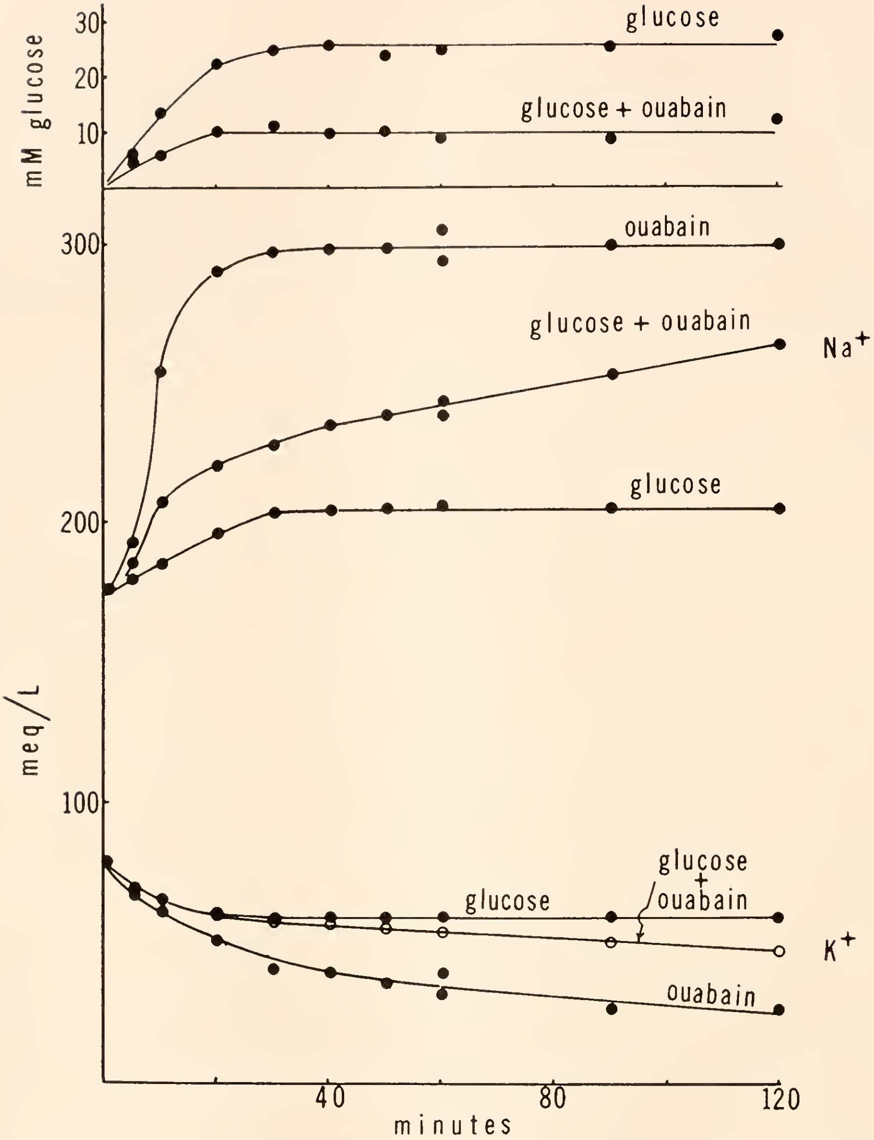

FIGURE 9. Effect of ouabain on Na^+ and K^+ net fluxes and on the accumulation of glucose. Glucose and ouabain at concentration of 5 m*M*. All values are expressed in terms of tissue water and each point is average of two samples. Controls incubated without glucose and/or ouabain showed no net change in Na^+ or K^+ levels during the same time period.

containing saline with or without 5 m*M* ouabain. The media were then assayed for Na^{24}. Results of such an experiment are shown in Figure 10. The data indicate that sodium effluxes from at least two compartments. Ouabain appears to exert an effect on one compartment and not on the other. Efflux from the

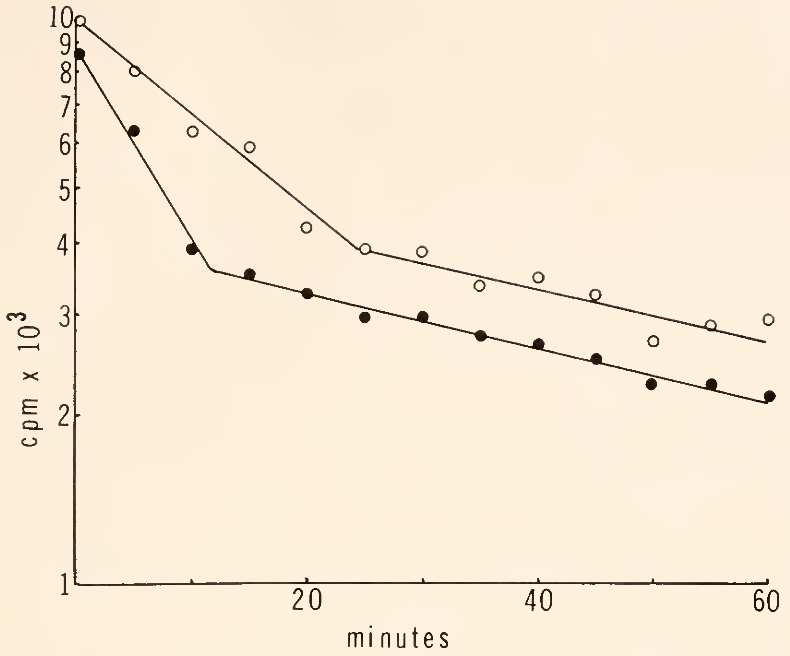

FIGURE 10. Effect of ouabain on efflux of Na^+. Worms were equilibrated for 60 min in saline containing Na^{24}. Efflux was then measured for 60 min in the presence (○) or absence (●) of 5 m*M* ouabain. See text for experimental details.

"fast compartment" is clearly inhibited by ouabain, whereas efflux rates from the other compartment appear to be the same for ouabain-treated and control samples.

Accumulation of other hexoses

Since glucose was accumulated against a concentration difference, it was of interest to determine whether some other hexoses are accumulated by *Calliobothrium* and to examine the relative rates at which these hexoses might be incorporated into parasite polysaccharide. Such data are presented in Table VI.

Of those examined, galactose and glucose are accumulated against a concentration difference. Although a specific galactose oxidase was not employed to examine the levels of galactose in the worm, the amount of non-glucose, ethanol-soluble carbohydrate (Table IV, column d) is about four times that found in worms incubated with other sugars. It is concluded that a large proportion of this non-glucose carbohydrate is indeed galactose. Although galactose is transported, very little of this sugar is incorporated into polysaccharide (Table IV, column g). Mannose and fructose are not transported and, as anticipated, negligible amounts of label from these sugars are incorporated into polysaccharide. Additional experiments on the absorption of 3–0-methylglucose showed that the rate is a linear function of sugar concentration and that it is neither accumulated against a concentration difference nor metabolized by the worm.

The data indicate that 3–0-methylglucose enters the worm by diffusion and substantiates the finding that this sugar does not react with the glucose transport system.

DISCUSSION

The outer surface of the syncytial tegument of *Calliobothrium* is elaborately differentiated for absorptive function, as is the case in all cestodes whose tegumentary ultrastructure has been studied (Lumsden, 1966). As we have shown, glucose is absorbed at high rates by the worm and, at concentrations which probably fall in the range normally encountered by the worm in its environment, *Calliobothrium* accumulates glucose against a concentration difference of up to 315 fold in a 30 minute period. The capacity of the worm to accumulate glucose is not affected by the presence of carbon dioxide or bicarbonate in the medium, although carbon dioxide clearly affects the rate at which glucose is incorporated into glycogen. This is similar to results obtained with the cyclophyllidean tapeworm *Hymenolepis diminuta* (Fisher and Read, unpublished data).

TABLE IV

Accumulation of radioactive hexoses by Calliobothrium *and incorporation into polysaccharide; incubation time: 60 min, incubation medium: 4.0 ml of 5.0 m*M *substrate; average of 2 samples/hexose; gas phase: 95% N_2–5% CO_2*

Subst.	C^{14} ("m*M*") (a)	Gluc. (m*M*) (b)	EtOH$^{sol.}$ CHO ("m*M*") (c)	Non Gluc. CHO ("m*M*") (d)	Medium Gluc. (m*M*) (e)	Medium Total CHO ("m*M*") (f)	Sp. act. Polysac. (g)
Gluc	46.78	31.23	38.60	7.37	3.84	3.94	0.1248
Gala	66.37	11.27	59.89	48.62	—	2.00	0.0011
Mann	2.16	12.94	24.67	11.73	—	5.01	0.0004
Lev	1.02	11.55	23.61	11.61	—	5.01	0.0001
	—	8.87	23.51	14.64	—	—	—

(a) Based on specific activity of medium.
(b) Determined by glucose oxidase.
(c) Determined by phenol: H_2SO_4.
(d) Determined by difference in, c – b.
(e) Determined by glucose oxidase.
(f) Determined by phenol: H_2SO_4.
(g) μMoles C^{14} substrate incorporated/μmole hexose in polysaccharide.

The temperature optimum for glucose absorption is perhaps slightly higher than that normally prevailing in the marine environment of the host. However, the body temperature of an active dogfish may be slightly higher than that of the surrounding sea water and it seems probable that the worm in its host would absorb glucose at something approaching the maximum rate at a given concentration.

There is a high correlation between the pH in that part of the spiral intestine inhabited by *Calliobothrium* and the pH values at which maximum rates of glucose absorption were observed. In the anterior part of the spiral intestine, not inhibited by the strobila of *Calliobothrium,* the prevailing pH would reduce glucose absorption by 30 to 35%. This might be of significance in allowing normal function of the worm. On the other hand, the free segments of *Calliobothrium* which are in varying stages of shelled egg development are typically found in the two anterior valves of the spiral intestine. The energy requirements of the free segments may be considerably less than the requirements of the strobila, since active tissue growth must be occurring at lower rates.

The inhibition produced by dinitrophenol clearly does not occur at the surface since it produced no inhibition in 2 min incubations. It may produce its effect over longer periods of exposure by general interference with water and salt movements. Iodoacetate, on the other hand, is a very non-specific poison and seems to produce an effect on the initial rates of glucose absorption probably combining with the carrier itself.

Of other potential inhibitors tested, only a limited number of hexoses and glycosides were inhibitory. None of the amino acids and several sugars were not inhibitory. Of the inhibitory sugars, five were found to produce inhibition in a competitive manner. The others were not examined with respect to this point. However, the observed competitive inhibitions of glucose uptake by other sugars; including the lack of inhibition by 3–0-methylglucoside and the inhibition by cellobiose and maltose suggest that the specificity of the site for glucose attachment to the transport mechanism in *Celliobothrium* differs from that in hamster mucosal cells (Crane, 1960) and in the tapeworm *Hymenolepis diminuta* (Read, 1961). Study of possible inhibitory effects of larger series of sugars, particularly hexoses, is required for definition of the points of glucose attachment in the transport mechanism. The lack of interaction by 3–0-methylglucose suggests that the third carbon on the hexose molecule is involved in glucose interaction. The finding that 3–0-methylglucose appears to enter the worm by diffusion, rather than by a mediated process, independently supports the concept that this sugar cannot react with the glucose transport system.

The finding that maltose and cellobiose inhibit glucose uptake must be interpreted with caution. It would be unwise to assume that the inhibitory effects are produced by the intact disaccharides. It is well known that disaccharidases are membrane-bound and act at the mucosal cell surface in the vertebrate intestine (Crane, 1968). The tapeworm surface has been described as a digestive-absorptive surface and, in the case of *Hymenolepis diminuta*, has been shown to release the products of hexose phosphate hydrolysis in the external medium (Arme and Read, 1970; Dike and Read, in press). Lumsden, Gonzalez, Mills and Viles (1968) also described an ATPase acting at the outer boundary of the tegument in *H. diminuta.* Further, the rabbit tapeworm *Cittotaenia* has a membrane-bound disaccharidase which hydrolyzes sucrose, liberating fructose into the external medium (Read and Rothman, 1958). Hence, there is a possibility that the apparent inhibition of glucose transport in *Calliobothrium* by maltose and cellobiose might be due to effects of glucose liberated by the action of intrinsic membrane-bound disaccharidases situated in close proximity to the glucose transport system in the outer face of the *Calliobothrium* tegument. The results of our experiments in which maltose in the external medium appeared to produce enhanced efflux of previously accumulated glucose might indicate the action of a surface maltase. In studies to be published elsewhere, Fisher has shown that *Calliobothrium* does not secrete a maltase into the external medium. Clearly, the inhibition of glucose transport by maltose and cellobiose requires further study.

Of the glycosides tested, phlorizin was the strongest inhibitor of glucose transport. Laurie (1961) reported that phloretin, the aglycone moiety of phlorizin, was a strong inhibitor of glucose absorption. We have not verified this. However, Laurie's experiments were of 1 to 4 hour duration, while those of the present work were 2 min incubations. Phloretin may affect glucose accumulation rather than the initial rate of glucose transport in *Calliobothrium.*

The changes in tissue sodium occurring with glucose absorption strongly suggest that glucose and sodium enter this worm by co-transport. Since sodium seems to activate glucose transport as an arithmetically linear function of sodium concentration, it may be concluded that reaction of only one sodium ion is required to activate the system for the transport of a glucose molecule. The data also suggest that a change in transmembrane electric potential should accompany glucose transport in *Calliobothrium,* but this has not been measured. The effects of ouabain on sugar transport and sodium fluxes furnish additional evidence that sodium is involved in glucose transport and glucose accumulation. The results seem to be completely consistent with Crane's view that glucose accumulation in vertebrate cells is attributable to the maintenance of low intracellular sodium, with a resulting lower affinity of the membrane carrier for sugar on the intracellular aspect of the cell boundary (Crane, 1965). In the present study, ouabain has been shown to cause an increase in tissue sodium and a decrease in glucose accumulation. The effect on sodium levels seems to involve inhibition of the sodium extrusion process since sodium efflux is clearly inhibited. The partial reversal of the ouabain effect on the net influx of sodium cannot be readily explained and would merit further investigation. While ouabain may inhibit glucose accumulation through effects on sodium extrusion, with a consequent lowering of the external/internal sodium ratio, there remains a problem in explaining the inhibition of glucose uptake by ouabain in 2 min incubations. This inhibition of the initial rate of glucose absorption seems to be competitive in nature, and it seems probable that ouabain interacts directly with the transport system.

It might be suggested that ouabain might be hydrolyzed with the release of a competitively inhibitory sugar. However, we found the glycone moiety, rhamnose, to be without effect on glucose absorption. Thus, we may postulate that, in *Calliobothrium,* ouabain inhibits sugar transport directly by interacting with the membrane carrier and inhibits sugar accumulation indirectly by inhibiting the sodium extrusion mechanism. Further work is required to substantiate this interpretation.

Sodium in the external medium is required for glucose absorption by both larvae and adults of the cyclophyllidean tapeworm, *Taenia taeniaeformis.* In the absence of sodium, no glucose transport occurs and, at 12 and 46 mM sodium, glucose absorption is reduced about 70 and 25%, respectively (von Brand and Gibbs, 1966). If the response to sodium concentration is linear, as is the case with *Calliobothrium,* about 60 mM sodium would be required to maintain glucose transport at its highest level in *T. taeniaeformis.* This has not been examined experimentally, although von Brand and Gibbs (1966) found that in media containing 115 mM sodium, glucose absorption equaled that observed in media containing 150 mM sodium. *Hymenolepis diminuta* also requires sodium for glucose transport. This will be discussed elsewhere (Fisher, in preparation).

The data on accumulation of various sugars by *Calliobothrium* verifies earlier reports (Read, 1957; Laurie, 1961) that this worm does not metabolize mannose or fructose. The worm is almost impermeable to these sugars. In the case of galactose, the worm seems to accumulate the sugar to higher levels than glucose can be accumulated. *Calliobothrium* has been reported to ferment galactose at rates almost equivalent to the rates of glucose fermentation (Read, 1957; Laurie, 1961). Thus, it may seem surprising that virtually no ^{14}C-galactose was incorporated into the polysaccharide of the worm. In a 60 min period, incorporation of ^{14}C-glucose into polysaccharide was 125-fold that of galactose. However, galactose is not glycogenic in the cyclophyllidean tapeworm *Hymenolepis diminuta*. Although starved *H. diminuta* showed a dramatic net glycogenesis when furnished with glucose, no significant net glycogenesis occurred when galactose was available (Read, 1967). Like *Calliobothrium, H. diminuta* transports galactose into the tissues by a specific mediated system and ferments this sugar, but galactose is not glycogenic.

A taxonomic note

Euzet (1954) reported that he had examined Linton's (1891) tapeworm material collected from dogfish at Woods Hole. Linton had identified these worms as *Calliobothrium eschrichtii* Beneden, but Euzet concluded that Linton's material represented a new species which he named *Calliobothrium lintoni*. However, the animal studied in the present investigation (as well as in previous studies by Read, 1957; Read *et al.*, 1960; Simmons, Read and Rothman, 1960; Laurie, 1961) is indeed *Calliobothrium verticillatum* (Rudolphi). We have found *C. lintoni* on several occasions in *Mustelus canis*. It is too small an organism for convenient physiological research.

SUMMARY

1. Gas phase had no significant effect on glucose absorption or accumulation by *Calliobothrium*.

2. Optimum temperature for glucose accumulation is about 20° C.

3. Glucose transport was affected by pH, highest rates being observed at 8 to 9. This corresponds to the pH measured in that part of the spiral intestine inhabited by the strobilate worm.

4. Those sugars or glycosides which inhibited transport of glucose also stimulated efflux. Inhibitions produced by phlorizin, ouabain, galactose, maltose, and α-methylglucoside were found to be competitive in 2 min incubations.

5. The rate of glucose transport was a function of Na^+ in the external medium. Tissue Na^+ increased and K^+ decreased during glucose absorption.

6. Ouabain caused a net influx of Na^+ and this effect was attributed to the observed inhibition of Na^+ efflux.

7. Galactose was also accumulated by the worm but a negligible amount was incorporated in polysaccharide. Mannose, fructose, and 3–0-methylglucoside were not transported.

8. The data are discussed in terms of the specificity of the glucose transport mechanism. It is hypothesized that ouabain may inhibit two processes involved in glucose transport and accumulation.

LITERATURE CITED

Arme, C., and C. P. Read, 1970. A surface enzyme in *Hymenolepis diminuta* (Cestoda). *J. Parasitol*, **56**: 514–516.

Brand, T. von, 1966. *Biochemistry of Parasites.* Academic Press, New York, 429 pp.

Brand, T. von, and E. Gibbs, 1966. Aerobic and anaerobic metabolism of larval and adult *Taenia taeniaeformis.* III. Influence of some cations on glucose uptake, glucose leakage, and tissue glucose. *Proc. Helminthol. Soc. Wash.*, **33**: 1–4.

Crane, R. K., 1960. Intestinal absorption of sugars. *Physiol. Rev.*, **40**: 789–825.

Crane, R. K., 1965. Na^+-dependent transport in the intestine and other animal tissues. *Fed. Proc.* **24**: 1000–1006.

Crane, R. K., 1968. A concept of the digestive absorptive surface of the small intestine. Pages 2535–2542 in C. F. Code, Ed., *Handbook of Physiology. Alimentary Canal, Sect. 6, Vol. 5.* American Physiological Society, Washington, D. C.

Dike, S. C., and C. P. Read, 1970. Tegumentary phosphohydrolases of *Hymenolepis diminuta. J. Parasitol.*, in press.

Dubois, M., K. A., Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**: 350–356.

Euzet, L., 1954. Quelques especes du genre *Calliobothrium* Van Beneden, 1850 (Cestoda: Tetraphyllidea). *Bull. Soc. Neuchâtel. Sci. Natur.*, **77**: 67–79.

Laurie, J. S., 1961. Carbohydrate absorption in cestodes from elasmobranch fishes. *Comp. Biochem. Physiol.*, **4**: 63–71.

Linton, E. (1891). Notes on entozoa of marine fishes of New England, with descriptions of several new species. Part II. *U. S. Comm. Fish Fish.*, **1887**: 719–895.

Lumsden, R. D., 1966. Cytological studies on the absorptive surfaces of cestodes. *Z. Parasitenk.*, **27**: 355–382.

Lumsden, R. D., G. Gonzales, R. R. Mills and J. M. Viles, 1968. Cytological studies on the absorptive surfaces of cestodes. III. Hydrolysis of phosphate esters. *J. Parasitol.*, **54**: 524–535.

McDaniel, J. S., C. P. Read and A. J. MacInnis, 1971. Some effects of carbon dioxide on glycogenesis in flatworms. *J. Parasitol.*, in press.

Phifer, K. O., 1960. Permeation and membrane transport in animal parasites: On the mechanism of glucose uptake by *Hymenolepis diminuta. J. Parasitol.*, **46**: 145–153.

Prichard, R. K., and P. J. Schofield, 1968. Phosphoenolypyruvate carboxykinase in the adult liver fluke, *Fasciola hepatica. Comp. Biochem. Physiol.*, **24**: 773–785.

Read, C. P., 1957. The role of carbohydrates in the biology of tapeworms. III. Studies on two species from dogfish. *Exp. Parasitol.*, **6**: 288–293.

Read, C. P., 1961. Competitions between sugars in their absorption by tapeworms. *J. Parasitol.*, **47**: 1015–1016.

Read, C. P., 1967. Carbohydrate metabolism in *Hymenolepis* (Cestoda). *J. Parasitol.*, **53**: 1023–1029.

Read, C. P., and A. H. Rothman, 1958. The role of carbohydrates in the biology of cestodes. VI. The carbohydrates metabolized *in vitro* by some cyclophyllidean species. *Exp. Parasitol.*, **7**: 217–223.

Read, C. P., and J. E. Simmons, Jr., 1963. Biochemistry and physiology of tapeworms. *Physiol. Rev.*, **43**: 263–305.

Read, C. P., J. E. Simmons, Jr., J. W. Campbell and A. H. Rothman, 1960. Permeation and membrane transport in animal parasites: Studies on a tapeworm-elasmobranch symbiosis. *Biol. Bull.*, **119**: 120–133.

Simmons, J. E., Jr., C. P. Read and A. H. Rothman, 1960. Permeation and membrane transport in animal parasites: Permeation of urea into cestodes from elasmobranchs. *J. Parasitol.*, **46**: 43–50.

Stein, W. D., 1967. *The Movement of Molecules across Cell Membranes. Theoretical and Experimental Biology, Vol. 6.* Academic Press, New York, 369 pp.

Reference: *Biol. Bull.,* **140**: 63–72. (February, 1971)

POPULATION AGE STRUCTURE, GROWTH AND LONGEVITY OF THE MARINE GASTROPOD *UROSALPINX CINEREA* SAY[1, 2]

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Urosalpinx cinerea Say is a West Atlantic temperate gastropod which is distributed on the North American coast from Nova Scotia to Nassau Sound, Florida. The species also occurs in the British Isles where it was probably introduced with consignments of the American oyster, *Crassostrea virginica* Gmelin (Orton, 1927). *U. cinerea* preys on a wide variety of marine invertebrates (Carriker, 1955; Wood, 1968) and in localities where the oyster is cultured, destruction of young oysters may be a serious commercial problem.

This species has been the subject of much research but information on its growth and longevity is incomplete, particularly for populations in North America. Information on the size of *U. cinerea* from different localities is available, notably the work of Cole (1942), Stauber (1943), Walter (1910), Federighi (1931a) and Myers (1965) and certain workers have attempted, unsuccessfully, to correlate size with temperature (Federighi, 1931b; Fraser, 1931). The problems involved in this type of correlation have been discussed by Carriker (1955) and Chestnut (1955).

The only detailed studies of growth of *U. cinerea* are based on populations from English waters (Cole, 1942; Hancock, 1959). Both authors assessed growth by size frequency analysis and Cole made use of growth interruption marks on the siphonal canal in evaluating the individual components of his polymodal size frequency curves. Andrews (1955) and others have commented that no evidence was provided to support the assumption that the interruption marks on the siphonal canal were annual growth marks (annuli). Andrews also noted that Cole's analysis lacks data on young *U. cinerea,* up through two years, owing to the difficulty in using an oyster dredge to collect small snails. Furthermore, Cole failed to provide evidence supporting his contention that the smooth curves fitted to the size frequency distributions represent year classes. Hancock's (1959) investigation included an analysis of size distribution of a population from the River Roach (Essex) as well as measurements of snails hatched and reared in the laboratory. By combining both sets of data, he was able to propose a cumulative growth curve for the population.

This investigation was undertaken to provide information on the age structure and growth of a North American population of *U. cinerea* (Southern New England) as a basis for future comparison with populations from other locations and ecological situations.

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METHODS

The subject of this study is a population of *U. cinerea* from the Mystic River, near Noank, Connecticut. The habitat is a rocky intertidal shore formed by a railroad embankment. The snails occupy approximately the lower third of the littoral zone where they occur on the sides and undersurfaces of rocks. Because of its location just within the mouth of the Mystic River, this shore habitat is well protected from wave action. The yearly salinity range is approximately 29–31 ppt and the seasonal temperature range is from −1–25° C. Ice forms on the shore in late winter.

Sampling was begun in June 1967 but for reasons noted below, most of this study is based on material collected in October, 1968, plus six samples from May through October, 1969. A total of approximately 2500 oyster drills was used in this analysis. The method followed in collection of the snails was as follows: collecting began about 30 minutes prior to LW and was continued for one hour. All snails were collected by hand by picking snails from individual rocks. Every rock selected was examined carefully to be certain that all snails were removed, thus reducing sampling bias in favor of large snails. The same stretch of shore and the same vertical level was sampled each time. Snails were taken to the laboratory for measurement and returned to the sampling location, usually the same day. In October, 1969, a supplementary sample of only larger drills was taken in order to increase the sample size of snails older than one season.

Shell height (distance from the tip of the siphonal canal to the top of the spire) to the nearest 0.1 mm was measured with vernier calipers, but snails under 8 mm were measured with a calibrated stereomicroscope. In addition to shell height, the shell weights were determined for one sample (October 1969) as follows: the snails were treated with concentrated potassium hydroxide for one week, rinsed several times with tap water and flushed with a pasteur pipette, over dried overnight at 65° C and weighed to the nearest 0.01 g.

The method of determining growth and longevity in this study is by size frequency analysis of both shell length and weight. An attempt has also been made to interpret and correlate growth interruption lines on the shell with the size frequency data. Difficulties encountered in this type of correlation in Mollusca have been discussed by Haskin (1954) and Wilbur and Owen (1964). A particularly vexatious problem in *U. cinerea* is caused by the extended reproductive period and consequent large range in size of each year class. The extensive overlapping between year classes, in conjunction with the lower growth rates of older oyster drills, makes the identification of older year classes always difficult and sometimes impossible. In the present investigation the problem is reduced by the simultaneous analysis of both shell length and shell weight.

RESULTS

Shell length frequency graphs for eight sampling dates beginning September 1967 are shown in Figure 1. The September 1967 population is polymodal but the lower peak, below about 8 mm, represents the 1967 year class which can be traced from its first appearance in the sample taken in July, 1967 (not shown). Sometime between September 1967 and the following June, an event important to the subsequent success of this study occurred, namely, the apparent disappearance of most of the 1967 year class. The cause remains unknown but the absence of this class is very evident in the June 1968 sample. The loss of most of this year class seems not to have seriously affected the population, as evidenced by the successful recruitment of the 1968 year class (Fig. 1, October 1968). The significance of the loss of the 1967 class becomes obvious in the May and June samples of 1969. In these, the total population is broadly bimodal. The lower (left) component clearly represents the previous (1968) year class; the upper component, a mixture of year classes including 1965 and 1966. The largely vacant space between these two components would have been occupied by the 1967 year class. Actually, a remnant of the 1967 year class is present and as shown below, can be demonstrated by the analysis of shell weight. In any case, the absence of most of the 1967 year class allows a more precise characterization of the 1968 class than would otherwise have been possible.

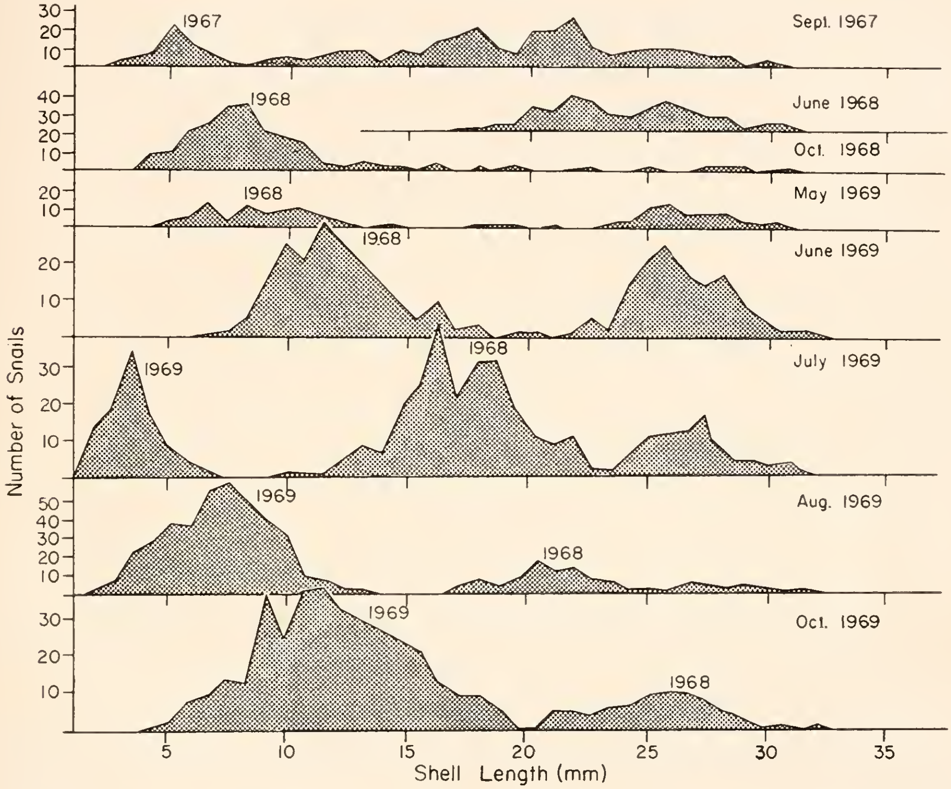

FIGURE 1. Size distribution of *Urosalpinx* from Noank, Connecticut, September 1967 through October, 1969. Numbers above the components of the distribution indicate the year of hatching.

In the July sample, the 1969 year class makes its first appearance. By August, the entire population consists of three components, the new 1969 class at the left, the 1968 class occupying the center of the distribution, and a component of one or more year classes older than 1968 at the right. Note that beginning in May of 1969, the 1968 class is persistently polymodal although this was not evident the previous October. The reason for this is not known but since there can be no doubt that only a single year class is involved, this year class has been treated as a single unit.

Upgrowth of the 1969 class is evident in the July through October samples. Growth of the 1968 class also continues so that by October 1969, the 1968 class merges with the remnants of the older classes.

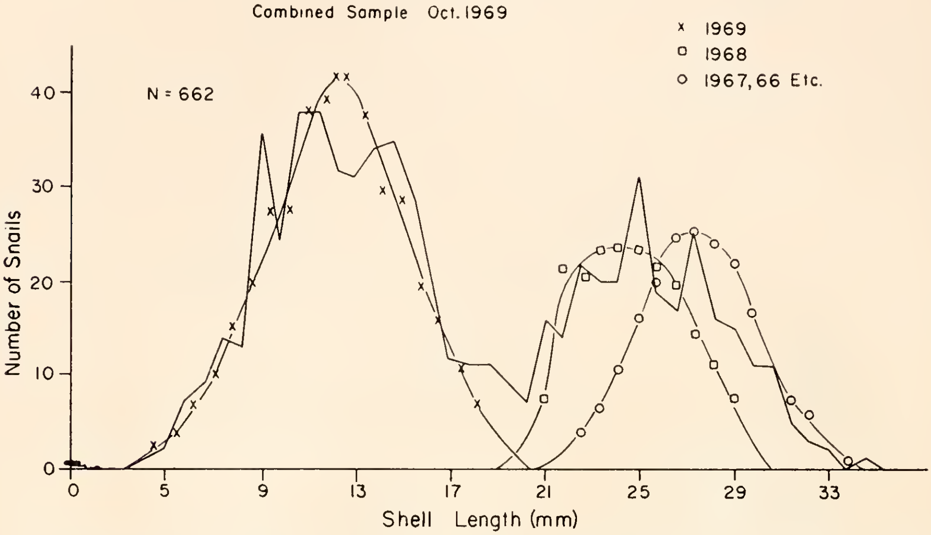

FIGURE 2. Size distribution of Noank *Urosalpinx*, pooled sample, October, 1969. Smooth curves have been fitted by the graphical "inflexion method" using normal probability paper (Lewis and Taylor, 1967).

Since the new class dominates the entire population in October 1969, a supplementary sample was taken the same day from which 1969 snails were excluded. Combined data from both of these samples is shown in Figure 2. The large component occupying the lower left of Figure 2 is unequivocally the 1969 year class. Using normal probability paper, this component was fitted with a smooth curve. The population component in Figure 2 above 20 mm is irregularly polymodal with two major peaks at 25 mm and 27.4 mm. Since this component must contain the 1968 class plus the population component representing the older classes, it is assumed that the peaks at 25 mm and 27.4 mm represent the modes of these two population components. Again, smooth curves were fitted to these two components. The three smooth curves of Figure 2 are postulations of the probably range, mode and degree of overlapping between the three population components present in the October 1969 sample. Two of these must correspond to the 1969 and 1968 year classes by virtue of their appearance and development in Figure 1. The third probably contains the remnant of the 1967 year class in addition to any older survivors.

The results of the shell weight analysis of the supplementary October sample are shown in Figure 3. The population is polymodal with at least four fairly well-defined components. These can be related to probable year class groups by comparison with Figure 2 and Figure 4, a double logarithmic regression of shell length and weight. The first component in Figure 3, with a mode of 0.22 g, includes oyster drills below about 20 mm and corresponds to the 1969 year class (some of which were inadvertently included in the supplementary October sample); the next component with a mode at 0.82 g corresponds to the 1968 year class; by a process of extrapolation, the third component probably represents the remnant of the 1967 year class. Figure 3 contains two further components, a larger with a major mode at 2.17 g which is interpreted as the 1966 year class, and a smaller number of even larger snails which may or may not be survivors of an older year class. It is evident from Figures 2 and 3 that the analysis of shell weight permits the discrimination of several size components not evident from shell length frequency alone.

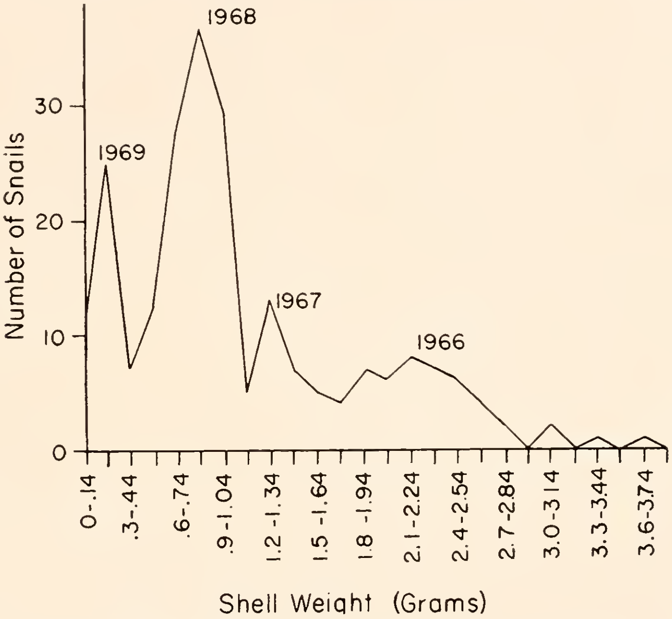

FIGURE 3. Shell weight distribution of supplementary 1969 sample. Numbers above the modes indicate the probable years of hatching.

Growth annuli

To find supporting evidence for the growth data provided by the size analysis, the drills comprising the supplementary October 1969 sample were examined for the presence of annual growth interruption marks (annuli) on the shell, the underlying assumption being that snails examined in October at the end of their first growing season should lack annuli. Snails completing their second growth season should have a single annulus, after three seasons a second annulus should be present, and so on. The problem is to discriminate between a true annual growth interruption mark and a mark resulting from some other cause such as damaged outer lip. Consequently, the decision as to whether a mark is indeed a true annulus is highly subjective and in many cases impossible. Drills which were damaged or in which the cause of the interruption was in doubt were not included, so that data in Table 1 represent 168 out of an initial 224 snails.

It is evident from the table that there is a close correlation between the mean weight of snails in each of the first four growth mark categories and the midpoints of weight components believed to correspond to the 1966 through 1969 year classes (Fig. 3). The correlation between the range in weight of each of the growth mark categories and the range of their respective year class components is also close, with the exception of the group of snails with two growth marks. The excessive scatter in this group probably results from failure to discriminate between true and false annuli. This is particularly difficult in older drills because of erosion of the spire and partial masking by subsequent shell growth of a portion of the earlier annuli.

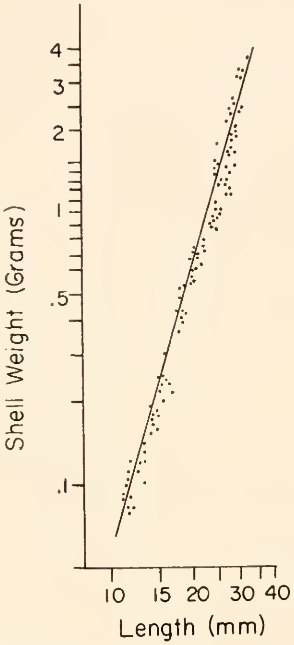

FIGURE 4. Double logarithmic plot of shell length and shell weight, pooled October 1969 sample. Curve is fitted visually.

Cumulative growth

Figure 5 is a cumulative growth curve based on the analysis of both shell weight and length. When the cumulative shell length is plotted against age, the expected sigmoid growth curve is produced with a point of inflection near

Table I

Weight frequency data for U. cinerea *possessing growth annuli*

Midpoint of weight interval (grams) (Modal groups are italicized)	No. snails per growth interruption category				
	"0 marks"	"1 marks"	"2 marks"	"3 marks"	"4 marks"
0.07	7				
0.22	21	3			
0.37	3	3	1		
0.52	0	7	0		
0.67	2	21	3		
0.82	0	28	3		
0.97	1	16	7		
1.12		4	1		
1.27		5	5		
1.42		1	1	1	
1.57		2	2	0	
1.72		1	0	0	
1.87			1	1	
2.02			2	2	1
2.17			0	2	0
2.32			2	2	0
2.47			1	1	0
2.62				2	1
2.77				1	1
2.92					
3.07					
3.22					
3.37					
3.52					
3.67					
Mean weight (grams)	0.22	0.82	1.26	2.22	2.47

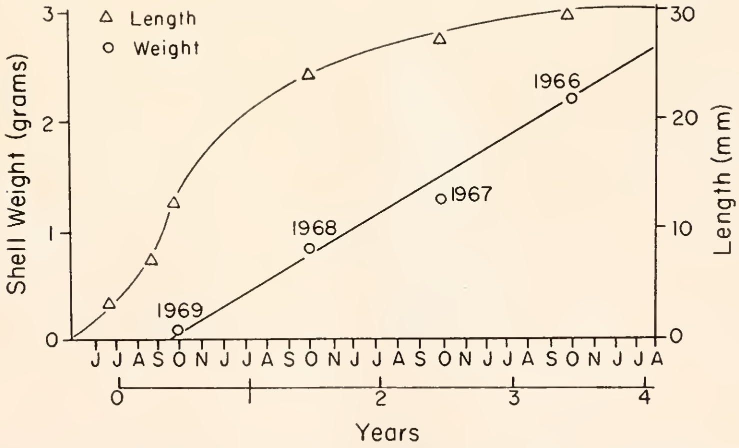

Figure 5. Cumulative growth curve for *Urosalpinx* from Noank, Connecticut. Letters stand for the months of the growth season, June through November.

the end of the first season, corresponding to the point of maximum growth rate. Close to seventy percent of the maximum shell length is attained by the end of the second growing season and little if any increase occurs after four years. The regression of shell weight on length approximates a linear function (Fig. 5), a characteristic which is shared by at least five other molluscan species (Sheldon, 1967). This author offered several possible explanations for this including the thickening of shell in older animals. In *U. cinerea* thickening of the outer lip occurs in older drills, as noted by Hancock (1959) and others.

Discussion

In general, the growth curve of Noank *U. cinerea* is similar to the River Roach (Essex) population reported by Hancock (1959), the only other population of the species for which comparable data are available. The population is comprised mostly of snails under 5 years of age and most of the shell growth occurs during the first two growing seasons.

The analysis of growth annuli provide evidence correlating these with size frequency. The usefulness of annuli in growth studies of *U. cinerea* is seriously hampered by the difficulty in discriminating between true and false annuli, especially when the latter are caused by damage to the outer lip early in the life of the snail. The growths marks discussed by Cole (1942) are useful but only in conjunction with growth annuli higher on the spire. Further studies using marked snails of known age would be helpful in delineating the variability in growth rates of the age classes as well as providing more precise information on population age structure.

One of the more interesting biological problems associated with growth of *U. cinerea* concerns the cause of the variation in maximum shell size which exists among populations. The most striking population in this regard occurs on the eastern shore of Virginia, particularly in Chincoteague Bay. The large size attained by individuals in this population is the basis for its designation as a subspecies, *U. c. follyensis* Baker (1951). However, even within the general range of shell size of *U. cinerea* there is considerable variability among populations. For example, there is some evidence that *U. cinerea* grows to a larger size in England than in North America (Fraser, 1931; Carriker, 1955; Cole, 1942) and on the Atlantic coast of the United States, significant variability occurs between populations (Fraser, 1931; Carriker, 1955; Cole, 1942).

While not excluding the significance of genetic factors in affecting maximum size, ecological factors acting on growth rate and the span of time during which growth continues are undoubtedly of great importance. These factors include the quality and quantity of available food and the length of the growing season. In the Noank population, remarkable year to year variability in mean shell size of the current year class is evident in the years 1968 and 1969 (Fig. 1). This variability seems to be related to certain ecological characteristics of this population.

Studies on the biology of *U. cinerea,* particularly those by Wood (1968), have shown that much of the biological success of this species can be credited to its capacity for utilizing a variety of prey species. The barnacle *Balanus balanoides* and the mussel *Mytilus edulis* are the major prey at intertidal habitats in the middle Atlantic region. At Noank, however, *Mytilus* is of no consequence as prey due to failure of this species to settle successfully at this site and there is no doubt that *B. balanoides* is the major prey. Predation on *B. balanoides* is intensive and snails in the process of attack have been observed many times, both in the field and laboratory. The barnacles settle in enormous numbers in the spring, usually March, so that a large prey population is already present when the snails emerge from dormancy in late April. Normally, however, only a single settlement of *B. balanoides* occurs so that predation by *U. cinerea* continually reduces a finite food supply. The age class of snails which most benefits from the large barnacle settlement is the previous year class, now in the second half of its first full year of life. The presence of a large food supply coinciding with a large component of the predator population capable of utilizing it may contribute to the high absolute growth rates which occur during the second half of the first year.

While *B. balanoides* is undoubtedly the major, and probably the preferred, prey species at Noank, it is not the only species preyed on by *U. cinerea*. Below LW, where barnacles are initially less dense than higher in the intertidal zone, the gastropod *Littorina littorea* is commonly attacked and it is not unusual to see several predators attacking a single *Littorina*. This may occur even when barnacles are available close by.

The encrusting ectoprocta, particularly *Cryptosula pallasiana* (Moll.), apparently occupy an important place in the ecology of *Urosalpinx* at this location. Although it is likely that all age groups feed to some extent on *Cryptosula*, newly hatched snails and snails still in their first season of growth are most frequently associated with the ectoprocts. This is understandable in view of the seasonal nature of the barnacle population. The latter settle in early spring. By the time the current year class of *U. cinerea* appears in July, the remaining barnacles ungrazed by older snails have grown significantly, decreasing their suitability as food for the newly hatched snails. The latter utilize the abundant supply of encrusting *Cryptosula*.

As noted by Wood (1968), it is unlikely that in the normal course of events *U. cinerea* ever completely decimates its supply of barnacles. However, there is also little doubt that as barnacles become less abundant due to grazing or other ecological factors, other species such as ectoprocts become increasingly more important as prey, especially in the lower portion of the intertidal zone. From an ecological point of view, this diversification in prey leads to a reduced competition for food within the population, *i.e.*, to a certain extent, newly hatched snails are not forced to compete directly with older members of the population for a single food supply. Although it is known that *U. cinerea* can undergo a normal, and even rapid, development in the laboratory when reared from hatching on a diet of oysters (Franz, 1966) or barnacles (Wood, 1968), ectoprocts occur abundantly virtually everywhere that *U. cinerea* is normally found. It would be interesting to determine if ectoprocts play a comparable role at other locations in the nurture of newly hatched *U. cinerea*.

SUMMARY

1. A study of growth and age structure in a Connecticut population of *Urosalpinx cinerea* was carried out using both shell length and weight frequency analysis. A cumulative growth curve is proposed and compared with other published data for the species.

2. Growth interruption marks (annuli) occur in *Urosalpinx* but are not as useful as in some other mollusks because of difficulty in discriminating annuli from interruptions caused by other factors.

3. Close to seventy percent of maximum shell growth is attained after two growing seasons. No increase in length occurs after four to five years and older snails, if present, constitute a very small proportion of the total population.

4. Growth of oyster drills in their first season is highly variable. Prey quality and availability may be the principal cause of this.

5. At Noank, *Balanus balanoides* is the major prey species of *U. cinerea* but newly hatched and very young snails frequently attack encrusting ectoprocta, particularly *Cryptosula pallasiana*. This diversification of prey is an advantage to the predator since it leads to a reduction in competition for food between juveniles and adults.

LITERATURE CITED

ANDREWS, J. D., 1955. Trapping oyster drills in Virginia. I. The effect of immigration and other factors on the catch. *Proc. Nat. Shellfish. Ass.*, **46**: 140–154.

BAKER, B. B., 1951. Interesting shells from the Delmarva Peninsula. *Nautilus*, **64**: 73–77.

CARRIKER, M. R., 1955. Critical review of biology and control of oyster drills *Urosalpinx* and *Eupleura*. *U. S. Fish Wildlife Serv., Spec. Sci. Rep. Fish.*, 148: 1–150.

CHESTNUT, A. F., 1955. The distribution of oyster drills in North Carolina. *Proc. Nat. Shellfish Ass.*, **46**: 134–139.

COLE, H. A., 1942. The American whelk tingle, *Urosalpinx cinerea* (Say) on British oyster beds. *J. Mar. Biol. Ass. U. K.*, **25**: 477–508.

FEDERIGHI, H., 1931a. Further observations on the size of *Urosalpinx cinerea* Say. *J. Conchol.*, **19**: 171–176.

FEDERIGHI, H., 1931b. Studies on the oyster drill (*Urosalpinx cinerea* Say). *Bull. Bur. Fish. U. S.*, **47**: 83–115.

FRANZ, D. R., 1966. Physiological variation among populations of the oyster drill, *Urosalpinx cinerea* Say. *Ph.D. thesis, Rutgers University*, New Brunswick, New Jersey, 194 pp.

FRASER, J. H., 1931. On the size of *Urosalpinx cinerea* (Say) with some observations on weight length relationships. *Proc. Malacol. Soc. London*, **19**: 243–254.

HANCOCK, D. A., 1959. The biology and control of the American whelk tingle *Urosalpinx cinerea* (Say) in English oyster beds. *Fish. Invest. London, Ser. II*, **22**(10): 1–66.

HASKIN, H. H., 1954. Age determination in mollusks. *Trans. New York Acad. Sci.*, **16**(6): 300–304.

LEWIS, T., AND L. R. TAYLOR, 1967. *Introduction to Experimental Ecology*. Academic Press, New York, 401 pp.

MYERS, T. D., 1965. A comparative study of size variation in the Atlantic oyster drill *Urosalpinx cinerea* (Say). *Masters thesis, University North Carolina*, Chapel Hill, North Carolina.

ORTON, J. H., 1927. The habits and economic importance of the rough whelk tingle (*Murex erinaceus*). *Nature*, **120**: 653–655.

SHELDON, R. W., 1967. Relationship between shell-weight and age in certain molluscs. *J. Fish. Res. Canada*, 24(5): 1165–1171.

STAUBER, L. A., 1943. Ecological studies on the oyster drill, *Urosalpinx cinerea*, in Delaware Bay, with notes on the associated drill *Eupleura caudata*, and with practical consideration of control methods. *Unpubl. Rept. Oyster Res. Lab., Rutgers University*, New Brunswick, New Jersey, 180 pp.

WALTER, H. E., 1910. Variations in *Urosalpinx*. *Amer. Natur.*, **44**: 577–594.

WILBUR, K. M., AND G. OWEN, 1964. Growth. Pages 211–242 in K. M. Wilbur and C. M. Yonge, Eds., *Physiology of Mollusca I*. Academic Press, New York.

WOOD, L., 1968. Physiological and ecological aspects of prey selection by the marine gastropod *Urosalpinx cinerea* (Prosobranchia: Muricidae). *Malacologia*, **6**(3): 267–320.

Reference: *Biol. Bull.*, **140**: 73–83. (February, 1971)

DEPOSITION OF CUTICULAR SUBSTANCES *IN VITRO* BY LEG REGENERATES FROM THE COCKROACH, *LEUCOPHAEA MADERAE* (F.)

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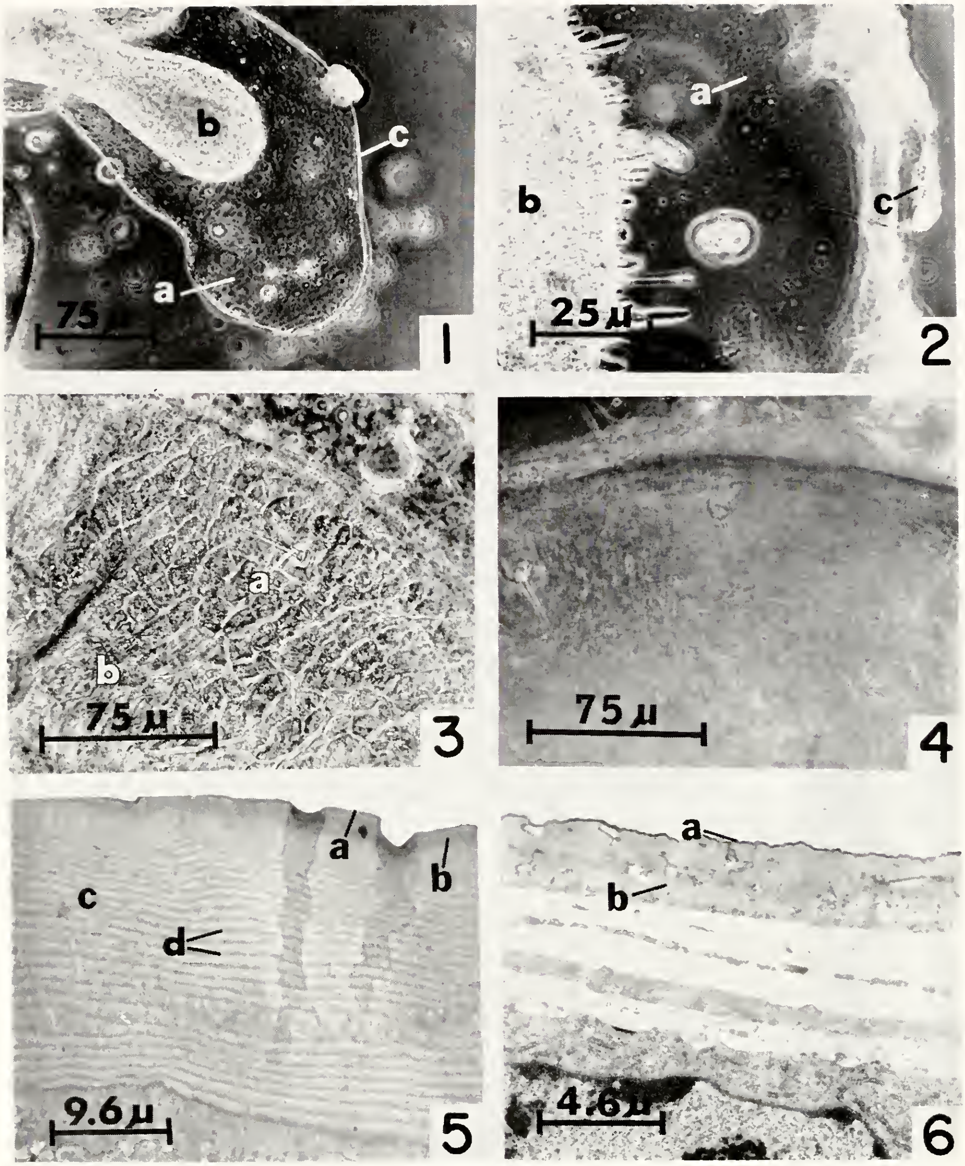
The appearance of cuticle-like membranes on the surface of insect tissues maintained *in vitro* has been reported by several investigators. Demal (1956) found a thin membrane covering explants of leg imaginal discs from 3-hour-old *Drosophila* prepupae held *in vitro*. He also observed thickening of the cuticle and the formation of tarsal claws. Sengal and Mandaron (1969) explanted leg imaginal discs of late-instar *Drosophila* larvae with the cephalic complex (including the ring gland); when the cultures were treated with ecdysone, many leg discs showed morphogenic changes that included the appearance of setae, surface scultpturing, and claws. Marks and Reinecke (1964) reported the secretion of a gelatinous material on the epidermis of leg regenerates of the cockroach, *Leucophaea maderae* (Fabr.), and Larsen (1966) cultured heart fragments from cockroach embryos and found a cuticle-like membrane covering vesicles that formed on these explants. Miciarelli Sbrenna, and Colombo (1967), working with explants of body wall from the abdomen of nymphs of the locust, *Schistocerca gregaria* (Forsk), found that the developmental stage of the donor insect greatly influenced the deposition of cuticle. Agui, Yagi, and Fukaya (1969) treated explants of epidermis from diapausing larvae of *Chilo suppressalis* (Walker) by adding small amounts of ecdysterone to the culture medium, and within 48 hours, the explant shed its old cuticle and deposited a new one.

Ritter and Bray (1968) cultured clots of blood from the cockroach, *Gramphadorina portentosa* in a medium that was free of insect plasma. The subcultures developed strands, flakes, and platelets around and within the clot, and the investigators concluded that the deposits were made up of a protein-chitin complex and that the complex was deposited on the surface of the culture vessel. However, the electron micrographic studies of Locke (1964) indicated that at least *in vivo*, the precursors are secreted by the epidermal cells.

Deposition of the cuticle in the regenerating leg of nymphs of the cockroach, *L. maderae*, was studied *in vivo* by Leopold and Marks (unpublished observations), and they found that two sheaths formed during regeneration. One appeared early in the development of the regenerate and thickened to form a gelatinous envelope around the regenerate. This layer apparently consisted of a protein-carbohydrate complex. The second sheath contained chitin, appeared late in development, thickened, and became wrinkled and rugose. The initial sheath was sloughed before molting, and the underlying chitin-bearing layer became the functional cuticle.



FIGURES 1–6.

FIGURE 1. A gelatinous sheath (a) laid down by the explant (b). The sharply defined outer border (c) is occasionally found in old, untreated cultures: *in vivo* 35 days: *in vitro* 53 days.

FIGURE 2. Gelatinous sheath (a) secreted by untreated explant (b) shows epithelial cells (c) extending into the sheath: *in vivo* 25 days; *in vitro* 40 days.

FIGURE 3. Detail of the surface of a mature leg regenerate that developed spontaneously *in vitro*. Seta (a) and surface sculpture (b) are characteristic: *in vivo* 35 days; *in vitro* 28 days.

In the present study, we have investigated some of the processes involved in cuticle secretion by combining the leg regenerate system with an *in vitro* methodology and histochemical analysis.

MATERIALS AND METHODS

Cockroach leg regenerates of different ages were prepared as described by Marks (1968). In the first tests, leg regenerates allowed to develop eight days after leg removal were dissected and placed under dialysis strips in Rose chambers with glass coverslips. The chemically defined M-7 medium was used. Six days later, they were examined with a phase contrast microscope, and the chambers were refilled with medium containing the test substance. After six more days, they were re-examined, and those showing cuticle-like deposits were scored as positive.

In the second test, leg regenerates were allowed to develop from 10 to 35 days after leg removal and were then dissected and examined. Those showing evidence of a cuticle were discarded, and the remainder were placed in Rose chambers between the dialysis strip and a glass coverslip. A plastic coverslip was added, and the chambers were filled with M-18 culture medium to which 5% fetal calf serum was added. The test was arranged with pairs of chambers, each containing a pair of leg regenerates from opposite sides of a single cockroach. One chamber of each pair was treated with ecdysterone, and the other was used as the control. Some of the controls were completely untreated, and others were treated with the inactive analog 22-iso-ecdysone (kindly supplied by Dr. John Siddall, Zoecon Corp., Palo Alto, California). The pairs were examined daily with a phase contrast microscope, and differences between the treated and untreated chambers were recorded. If the control showed evidence of developing cuticle, the treated chamber paired with it was removed from consideration. After 15 days, pairs in which the test leg developed a cuticular deposit were separated, and the test leg was discarded. Then the control was given 5 to 10 μg of ecdysterone to prove that its failure to produce cuticle had occurred because of the absence of the hormone and not from other causes.

The presence of chitin in selected specimens was verified by using the fluorescent enzyme technique of Benjaminson (1969). Frozen sections of the leg regenerate tissue were treated with chitinase conjugated with the fluorescent dye lissamine rhodamine B 200 chloride. They were examined by fluorescence microscopy, and fluorescence in cuticular structures was accepted as evidence of chitin.

RESULTS AND DISCUSSION

Deposition of the sheath

Leg regenerates that were allowed to develop for eight days before explanation normally showed no cuticle-like deposits when they were explanted. However,

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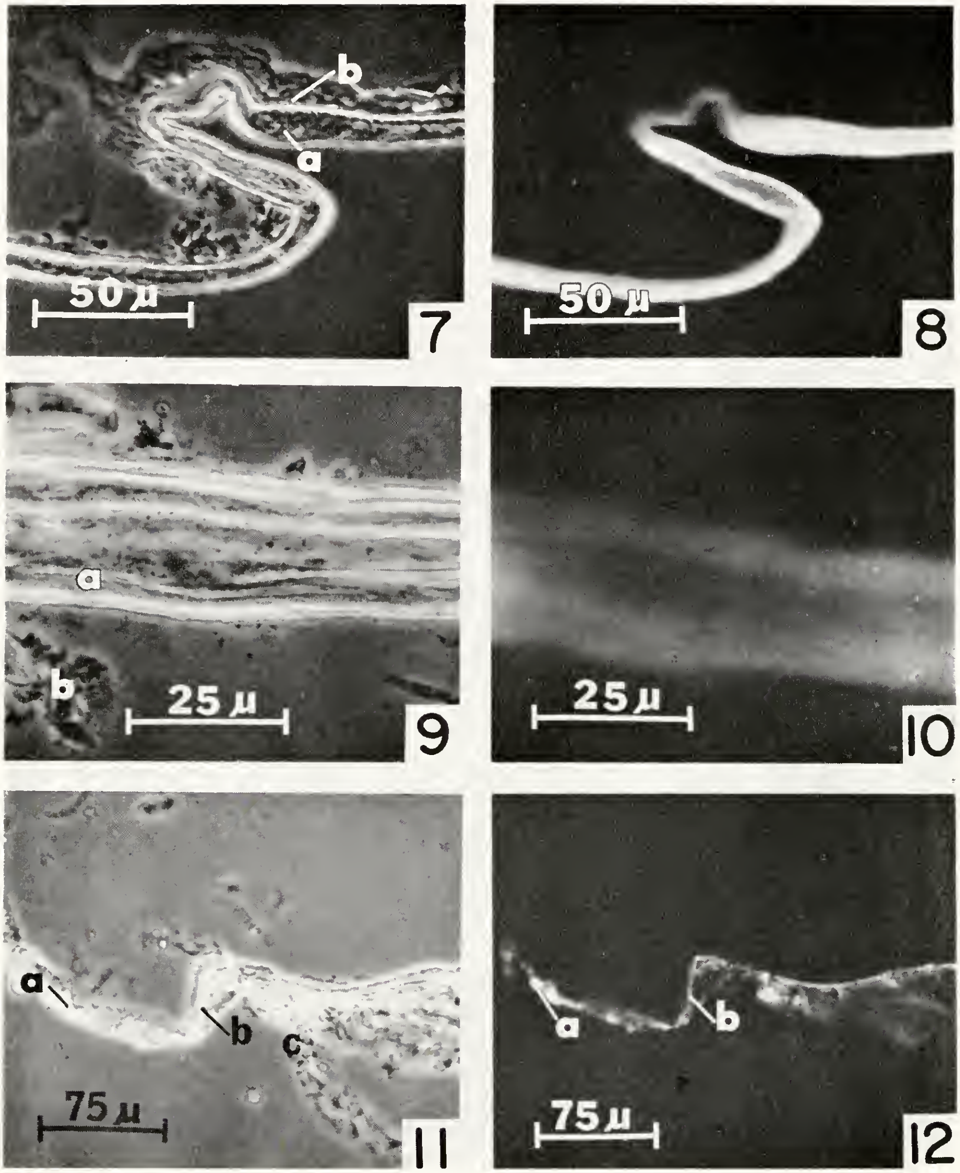
FIGURE 4. Detail of the surface of a 10-day-old leg regenerate treated with 12 μg/ml of ecdysterone. Setae are present but surface sculpture is absent: *in vivo* 10 days; *in vitro* 18 days.

FIGURE 5. Electron micrograph of cuticle from the leg of a newly molted cockroach. Epicuticle (a), sclerotized layer (b), and endocuticle (c) are clearly visible. Laminae (d) of the endocuticle are well formed and compact.

FIGURE 6. Electron micrograph of cuticle formed by a 25-day-old leg regenerate *in vitro* shows well-formed epicuticle (a) but poorly formed endocuticle (b): *in vivo* 25 days; *in vitro* 30 days.



FIGURES 7–12.

FIGURE 7. A section through cuticle from leg of freshly molted cockroach. This specimen is shown under phase contrast illumination. Cuticle (a), which is refractible, is underlain by the epidermis (b).

FIGURE 8. Same specimen as figure 7 subjected to Benjaminson chitinase procedure and fluorescent microscopy. The chitinase-dye complex that has conjugated with the cuticle fluoresces. Note that the epidermis does not conjugate with the enzyme-dye complex and is no longer visible.

when they were held *in vitro*, 13 per cent produced deposits of gelatinous material within 14 days. The deposit appeared as a thin transparent layer that gradually thickened (Figs. 1 and 2); also, flakes and threads of darker material and occasional layering of this material appeared. As the cultures aged, the outer border of the layer often became sharply defined and yellowish (Fig. 1). No evidence of definitive cuticle appeared even though some cultures were held as much as 120 days. Fluorescent chitinase tests showed that the gelatinous layer, which probably represented the protective sheath seen in *in vivo* preparations, was devoid of chitin.

The protective role of this sheath, which may be related to wound cuticle (Sannasi, 1968), is supported by the finding that methanol (0.5 μl/ml of medium) and incubates of muscle tissue were as effective in inducing sheath deposition as were ecdysterone (2.5 μg/ml of medium) and incubates of the prothoracic gland. This sheath material is probably the same as the gelatinous substance reported by Marks and Reinecke (1964) and may also be the same as the cuticular material reported by Larsen (1966).

Deposition of the cuticle

The first evidence of the deposition of the cuticle *in vitro* was the appearance of a thin refractile layer between the epidermal cells and the protective sheath. As the deposition continued, the epidermal cells rounded up, contracted, and produced a pebbled appearance. Parallel ridges appeared and increased in size and number so the entire surface of the explant had a rugose appearance. Setae formed from hair-like trichogen cells that protruded from the explant, and the tormagen cell became embedded in the surface and sclerotized (Figs. 3 and 4). The deposition of the cuticle was usually terminated by the withdrawal of the epidermal cells from the secreted structures and the development of a space between them. This process apparently represents the *in vitro* counterpart of apolysis.

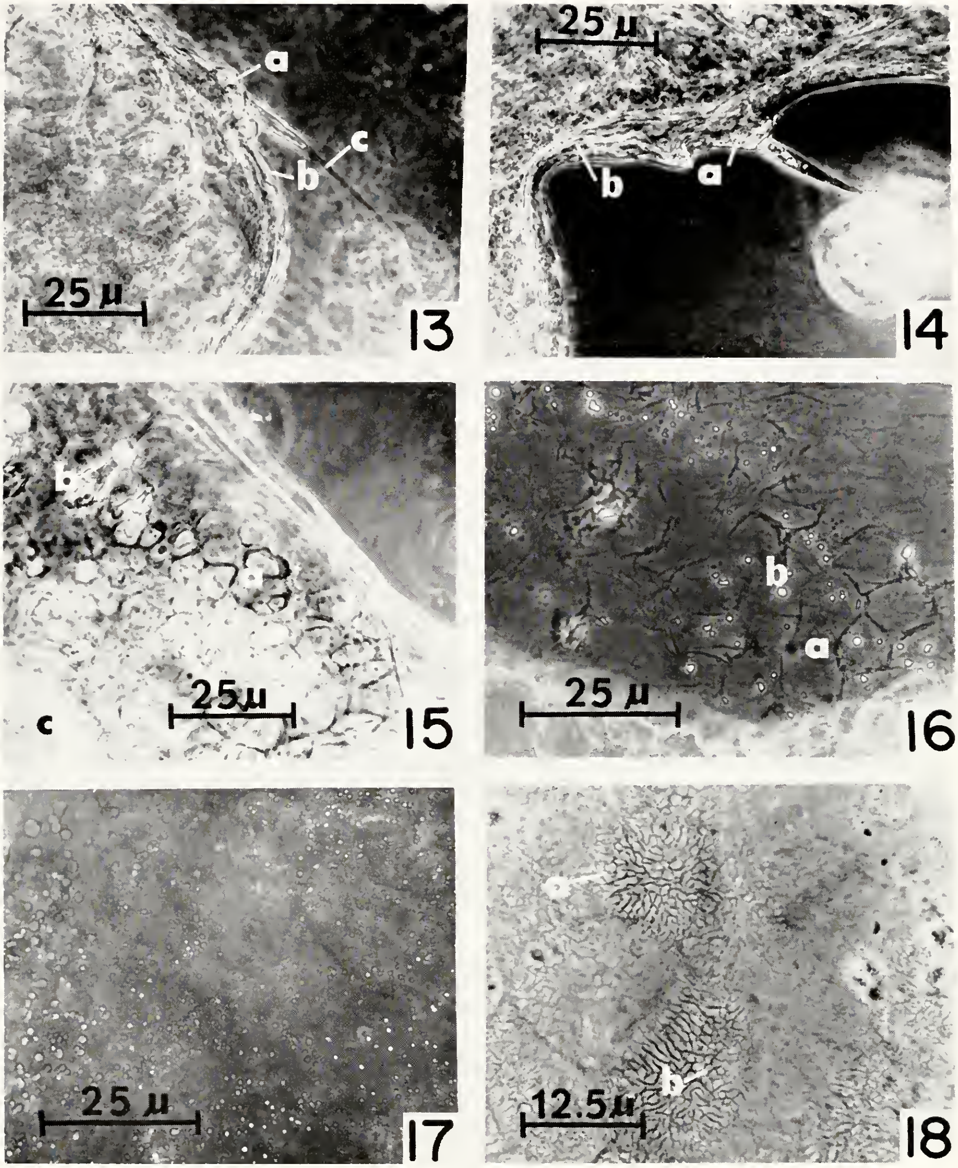
Examination of the ultrastructure showed that the cuticle regenerated *in vivo* possessed a thick endocuticle at the time of molting, and that the lamellae were compact and well formed (Fig. 5). Cuticle that formed *in vitro* (Fig. 6) showed a well developed epicuticle, but the endocuticle was only partially formed and not as compact as cuticle formed *in vivo*; separation of the cuticle from the epidermis was apparent.

FIGURE 9. Section through cuticle (a) formed by a 25-day-old leg regenerate *in vitro* seen with phase contrast illumination. Epidermis (b) has been largely stripped away in sectioning. The membrane is folded so the two layers are visible: *in vivo* 25 days; *in vitro* 20 days.

FIGURE 10. The same specimen as in Figure 9 subjected to the Benjaminson chitinase procedure and viewed by fluorescent microscopy shows two layers of fluorescence. Note fluorescence is not as intense as in Figure 8.

FIGURE 11. A section through cuticle formed *in vitro* by a 10-day-old leg regenerate viewed under phase contrast illumination. Note refractile droplets (a) appearing between the cuticle (b) and epidermis (c): *in vivo* 10 days; *in vitro* 21 days.

FIGURE 12. Same specimen as in Figure 11 subjected to the Benjaminson chitinase procedure and viewed by fluorescent microscopy. Both droplets (a) and solid cuticle (b) fluoresce, indicating the presence of chitin.



FIGURES 13–18.

FIGURE 13. A leg regenerate treated with 5 µg/ml of ecdysterone shows seta formation. Base of seta lies in the socket (a) that protrudes from the surface of the cuticle (b). The unsclerotized tip of the trichogen cell (c) is visible: *in vivo* 25 days; *in vitro* 25 days.

FIGURE 14. Another area of the same leg regenerate shown in Figure 13 shows two setae with well-formed sockets (a) embedded in cuticle (b). Only the base of the seta is sclerotized; upper end remains membranous: *in vivo* 25 days; *in vitro* 25 days.

FIGURE 15. Reticular pattern (a) and abnormal setae (b) are present on the surface of cuticle developing *in vitro*. Epidermis (c) has withdrawn, leaving the cuticle behind: *in vivo* 25 days; *in vitro* 25 days.

The dense cuticle formed *in vivo* demonstrated a strong reaction for chitin (Figs. 7 and 8) when it was tested by the fluorescent chitinase procedure. When the cuticle that formed *in vitro* was tested by this method, the reaction was weak but consistently positive (Figs. 9 and 10). The lower intensity of the fluorescence probably resulted from the lower density of the endocuticle in the *in vitro* preparations.

In some specimens, particularly among the younger explants, droplets of hyaline material often accumulated between the developing cuticle and the surface of the epithelial cells. When one such explant was sectioned and tested for chitin, we found a pebbled inner surface that contained chitin in the form of droplets (Figs. 11 and 12). Such droplets appeared only after treatment with ecdysterone and were most commonly found when the dosage was very low. The development of setae *in vitro* occurred with greatest frequency in explants that were taken from older nymphs (Table I). In some cases, the socket formed on the surface of the cuticle (Fig. 13) rather than imbedded in it (Fig. 14), and generally the sclerotization was incomplete.

We occasionally found cuticle forming over the surface of a vesicle, and when the vesicle later collapsed and withdrew the delicate epithelium, the remaining cuticle was left with a pattern representing the outline of the cells that deposited it (Figs. 15 and 16). The presence of the hyaline droplets often made it difficult to observe these patterns, but in those areas where they were absent, a pattern of ridges within the area laid down by a single cell was seen (Fig. 18). This apparently represented the imprint of the surface of the cell itself.

When leg regenerates were dissected late in the molting cycle (35 days), a large number had already initiated cuticle development *in vivo*. Those that had not were placed *in vitro*, and more than half continued their development without stimulation (Table I). Therefore, meaningful studies of the induction of cuticle development could only be made by using paired chambers containing legs from opposite sides of the same insect.

In an attempt to simplify our procedures and to increase our efficiency, we made a study of younger leg regenerates. Paired chambers were used, and leg regenerates from 5- to 30-day-old were treated with different doses of ecdysterone. The results indicated that while all doses above 2 μg/ml had about the same effect on cuticle production, the frequency with which the leg regenerates responded to stimulation increased with age (*in vivo*) until a 100 per cent response was reached at 20 days. There was also a corresponding increase in the frequency of seta formation with age.

The increase in ability to respond to the hormone with increased age seemed to continue after the tissue was placed *in vitro*. Thus, 10-day-old legs treated

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FIGURE 16. Reticular pattern (a) is visible in this very thin cuticle. Droplets (b) probably containing chitin (Figure 11) are present. Epidermis has withdrawn: *in vivo* 25 days; *in vitro* 10 days.

FIGURE 17. Detail of the surface of a developing cuticle shows droplets accumulating between the cuticle and the epidermis. Droplets probably contain chitin; *in vivo* 10 days; *in vitro* 18 days.

FIGURE 18. Surface detail of developing cuticle shows polygonal outlines of cells (a). Within the outlines is a pattern (b) that represents the negative image of the irregular surface of the cell. This may represent the first step in the formation of the surface sculpture: *in vivo* 25 days; *in vitro* 10 days.

four days after explanation gave a 20 per cent (2/10) response, but when the water controls for this series were treated with ecdysone 14 days later, there was a 66 per cent (4/6) response. A similar but less marked effect was obtained with 15-day-old leg regenerates. The untreated controls did not produce cuticle.

A second test was set up to determine the minimum dose of ecdysterone that would produce a 100 per cent response in 25-day-old regenerates. Paired chambers were not used because regenerates of this age did not spontaneously produce cuticle under experimental conditions. In the 12 specimens treated with 2 μl of water and in the 10 specimens treated with 24 μg/ml of 22-iso-ecdysterone, there was no development of cuticle; all specimens developed cuticle when they were later treated with 10 μg/ml of ecdysterone for 25 hours. In the experimental chambers, doses of ecdysterone in water ranging from 2.5 μg/ml of nutrient to 0.05 μg/ml were given; then after seven days, the chambers were emptied and refilled with fresh nutrient, and the date when cuticle deposition first appeared was recorded. A 100 per cent response was obtained with 2.5 μg/ml, a 90 per cent response was obtained with doses down to 0.5 μg/ml, a 37 per cent response was obtained with a dose of 0.2 μg/ml, and no response was obtained with a dose of 0.1 μg/ml or less. All the controls that were later exposed to the hormone developed cuticle.

TABLE 1

The effect of age at time of explanatation on development of cuticle in cockroach leg regenerates in vitro

Days after leg removal	Number tested	Per cent developing without treatment	Number (and per cent) developing after treatment with 2–10 μg/ml ecdysterone	
			Cuticle	Setae
10	10	0	2 (20)	1 (10)
15	8	0	7 (88)	1 (12)
20	6	0	6 (100)	2 (33)
25	16	0	16 (100)	10 (62)
30	4	0	4 (100)	3 (75)
35	5 (11)*	54	5 (100)	3 (60)

* Eleven were set up, and six (54%) developed spontaneously. All five remaining regenerates developed cuticle when treated with ecdysterone.

The appearance of setae and surface sculpture and the presence of chitin indicated that the cuticular deposits formed *in vitro* in response to stimulation by ecdysterone are the same as those deposits that are present *in vivo* at the time of molting. Thus, it is likely that the structures reported by Demal (1956) and Sengel and Mandaron (1969) as appearing on imaginal leg discs taken from late-instar *Drosophila* larvae probably also contained chitin. In Demal's studies, development was spontaneous since stimulation occurred before the leg imaginal discs were explanted, but evaluation of the results of Sengel and Mandaron is more difficult because the brain and ring gland were present in the same cultures as the imaginal discs and because development occurred in some of the carrier-control cultures. Interaction between the glands and the ecdysone cannot be ruled out since Burdette, Hanley, and Grosch (1968) reported that the effect of ecdysone on the ocular imaginal discs of *Drosophila* was enhanced by the presence of the cephalic lobes in the culture, and Williams (1952) showed that ecdysone can stimulate secretion by the prothoracic glands. However, the amount of ecdysone used by Sengel and Mandaron in the culture medium would probably have been sufficient to induce cuticle secretion by the leg imaginal discs, even in the absence of the gland explants. The work of Agui *et al.* (1969) further demonstrated that the entire process of molting, including the shedding of the old cuticle and the deposition of the new, can be induced in short-term cultures by adding ecdysterone to the culture medium.

In leg regenerates, the deposition of endocuticle *in vitro* is usually incomplete and varies considerably from one specimen to another. In general, the 35-day-old leg regenerates that produced cuticle *in vitro* tended to produce heavier deposits than did the 10- to 20-day-old ones. Two explanations for this are offered: The *in vitro* conditions may have been such that the normal synthesis of the endocuticle by the epithelial cells could not occur; therefore, the cuticle secreted *in vitro* would consist primarily of the epicuticle and that portion of the endocuticle secreted before the synthesis mechanism ceased to function. The other possibility, suggested by the experiments of Philogene and McFarlane (1967), is that the epithelial cells merely lay down a material that is either produced elsewhere or whose precursors are produced elsewhere. Then, by isolating the leg regenerate, we cut off the source of supply of this material except for that amount explanted along with the leg regenerate. This amount would be greater in regenerates explanted later in the molting cycle.

The fact that a 24-hour exposure of the leg regenerate to 10 μg/ml of ecdysterone was sufficient to induce cuticle deposition a week later suggests that the so-called "endogenous hormone" present in explants taken from insects late in the molting cycle probably refers to the physiological state of the tissue rather than to the actual presence of the hormone in the tissue. The fact that 10-day-old leg regenerates (which are little more than blood-filled sacs of epithelial tissue) can be induced to produce a seta-bearing cuticle with relatively small doses of ecdysterone indicates that this physiological state depends on the action of the hormone as well as on the age of the tissue. In *Leucophaea*, removal of a leg late in the stadium when the ecdysone titer is presumably high resulted in the formation of a cuticle-covered papilla, and regeneration of the leg resumed only after the subsequent molt. This *in vivo* response closely resembeled the response produced *in vitro* by treatment of 10-day-old leg regenerates with ecdysterone in which premature cuticle deposition eventually terminated morphogenesis.

However, if the leg was removed earlier in the cycle and allowed a longer period of development, a complete leg formed, and the approach of molting merely speeded up and completed the process. Similarly, Postlethwait and Schneiderman (1970) showed that when leg imaginal discs of *Drosophila* cultured *in vivo* were treated with large doses of ecdysterone, metamorphosis complete with formation of pupal cuticle occurred.

In our studies, we found that cuticle deposition can be stimulated by small doses of hormone presumably because other tissues (blood and fat body) are not present to inactivate the residual hormone and the target tissue is thus exposed to low levels of the hormone over a period of several days. In *in vivo* cultures, very large doses of the hormone were required to produce such an effect, thus suggesting that there may be a relationship between the amount of hormone present and the length of time that the tissue is exposed to it.

Ohtaki, Milkman and Williams (1968) proposed that tissue competence results from the accumulation of physiological events caused by continuous exposure to a low titer of hormone over a long period. It is just such a situation that we reproduced *in vitro, i.e.*, a low dose (0.2 μg/ml) of hormone left in contact with the target organ over a long period (7 days). Our results tend to substantiate the hypothesis of Williams' group. Furthermore, it should be possible to use this experimental system as a basis for a series of time-dosage studies that would shed additional light on this question.

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SUMMARY

Two types of sheath material are deposited during the development of leg regenerates in the cockroach, *L. maderae*. The first sheath to appear resembles wound cuticle because it contains no chitin. Its deposition *in vitro* can be induced by a number of unrelated substances. The second sheath contains chitin, bears spines and setae, and represents the cuticle. Deposition of this sheath is induced *in vitro* by microgram quantities of ecdysterone but not by other substances that were tested.

Leg regenerates dissected as early as 10 days after leg removal were capable of depositing cuticle, but a 100% response to stimulation with ecdysterone was obtained only with leg regenerates 20 or more days old.

LITERATURE CITED

Agui, N., S. Yagi and M. Fukaya, 1969. Induction of moulting of cultivated integument taken from a diapausing rice stem borer larva in the presence of ecdysterone. *Appl. Entomol. Zool.*, **4**: 156–157.

Benjaminson, M. A., 1969. Conjugates of chitinase with fluorescine isothiocyanate or lissamine rhodamine as specific stains for chitin *in situ*. *Stain Tech.*, **44**: 27–31.

Burdette, W. J. E. W. Hanley and J. Grosch, 1968. The effect of ecdysones on the maintenance and development of ocular imaginal discs *in vitro*. *Tex. Rep. Biol. Med.* **26**: 173–180.

Demal, J., 1965. Culture *in vitro* d'ebauches imaginales de Dipteres. *Ann. Sci. Natur. Zool. Biol. Anim.*, **18**: 155–161.

Larsen, W., 1966. Growth in an insect organ culture. *J. Insect Physiol.*, **13**: 613–619.

Locke, M., 1964. The structure and function of the integument in insects. Pages 370–470 in M. Rockstein, Ed., *The Physiology of Insecta, Volume III*. Academic Press, New York.

Marks, E. P., 1968. Regenerating tissues from the cockroach, *Leucophaea maderae*: The effect of humoral stimulation *in vitro*. *Gen. Comp. Endocrinol.*, **11**: 31–42.

MARKS, E. P., AND J. P. REINECKE, 1964. Regenerating tissues from the cockroach leg: A system for studying *in vitro*. *Science,* **143**: 961–963.

MICIARELLI, A., G. SBRENNA AND G. COLOMBO, 1967. Experiments of *in vitro* cultures of larval epiderm of desert locust. *Experientia,* **23**: 64–65.

OHTAKI, T., R. MILKMAN AND C. WILLIAMS, 1968. Dynamics of ecdysone secretion and action in the fleshfly, *Saracophaca peregrina*. *Biol. Bull.,* **135**: 322–334.

PHILOGENE, B., AND J. MCFARLANE, 1967. The formation of the cuticle in the house cricket. *Acheta domestica,* and the role of oenocytes. *Can. J. Zool.,* **45**: 181–190.

POSTLETHWAIT, J. H., AND H. SCHNEIDERMAN, 1970. Induction of metamorphosis by ecdysone analogues: *Drosophila* imaginal discs cultered *in vivo*. *Biol. Bull.,* **138**: 47–55.

RITTER, H., AND M. BRAY, 1968. Chitin synthesis in cultivated cockroach blood. *J. Insect Physiol.,* **14**: 361–366.

SANNASI, A., 1968. Hyaluronic acid in the scar tissue of cockroach. *Zool. Jahrb. Abt. All. Zool. Physiol. Tiere,* **74**: 319–327.

SENGAL, P., AND P. MANDRARON, 1969. Aspectes morphologiques du developpement *in vitro* des disques imaginaux de la *Drosophile*. *C. R. Acad. Sci. Paris,* **268**: 405–407.

WILLIAMS, C. M., 1952. Physiology of insect diapause IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull.* **103**: 120–138.

Reference: *Biol. Bull.*, **140**: 84–94. (February, 1971)

BIOLOGY AND LIFE HISTORY OF THE NUDIBRANCH MOLLUSC, *CORYPHELLA STIMPSONI* (VERRILL 1879)

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Verrill (1879) originally described *Coryphella stimpsoni* from Eastport, Maine and in 1880 reported its range from Massachusetts Bay to Halifax, Nova Scotia. Bergh (1885) recorded the morphology of *C. stimpsoni* based on a preserved specimen collected in Nova Scotia and sent to him by Verrill. Krause (1892) and Knipowitsch (1902) found *C. stimpsoni* in Northern Europe and the latter described a new variety based on radular variation.

In October, 1968 and 1969 a large number of *Coryphella stimpsoni* were encountered moving over mud flats in what appeared to be a mass migration at the Moosehorn Wildlife Refuge, Edmunds, Maine. The nudibranchs were taken to the Northeastern University Marine Science Institute where further observations were made which are reported here.

MATERIALS AND METHODS

Observations and measurements were made on living animals whenever possible. Radular mounts were prepared utilizing Turtox CMCS and Gomori trichrome stain; the latter one clearly differentiated the teeth from the radular membranes.

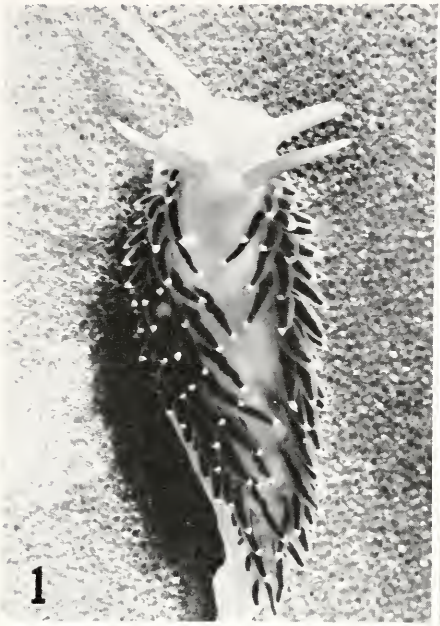
At the Marine Science Institute, the specimens were maintained in plexiglass tanks. Egg masses were isolated in glass dishes on the water table. Newly hatched specimens were relaxed in 5% M_gCl_2, fixed in Hollande's fluid, sectioned and stained. Adult specimens were relaxed in 8% M_gCl_2, fixed in Hollande's fluid and 10% formalin. Representative specimens have been deposited at the U. S. National Museum (Smithsonian) and in the Mollusk Department of the Museum of Comparative Zoology at Harvard University.

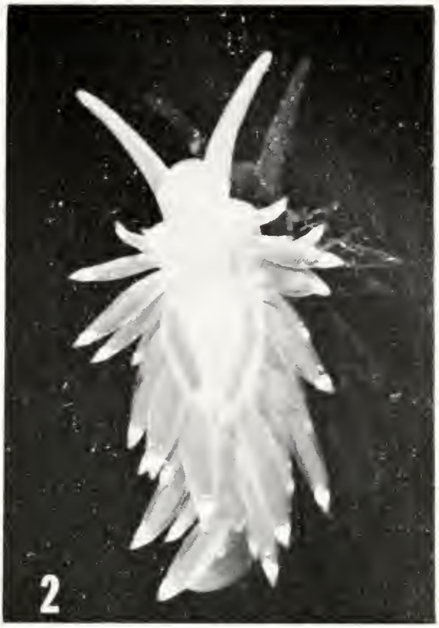
RESULTS

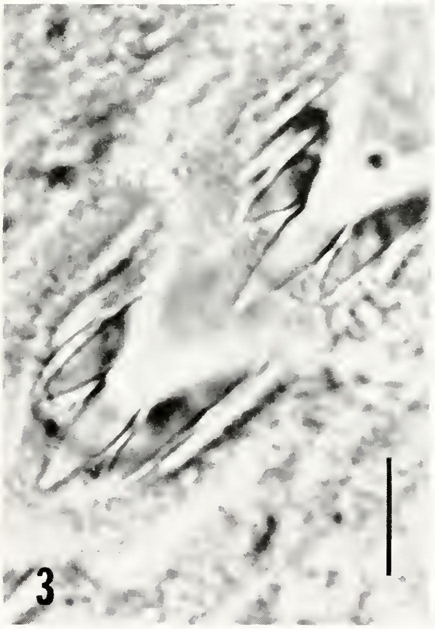
Description of the adults

Living specimens ranged from 13–20 mm in length and the foot measured 4.5–5 mm in greatest width. The cerata are evenly distributed in two longitudinal bands on the dorsal surface and extend anteriorly beyond the position of the paired rhinophores. The cerata are largest medially and decrease in size while increasing in number toward the lateral margin of the mantle (Fig. 1).

The head is large and forms a characteristic trefoil due to the presence of large rounded lateral lobes. The color of the head and tentacles is an opaque white, identical to the body color. The smooth oral tentacles are slightly longer, taper more abruptly, are wider at the base and narrower at the tip than the rhinophores. During movement, the tentacles are continually brought in contact with the sub-







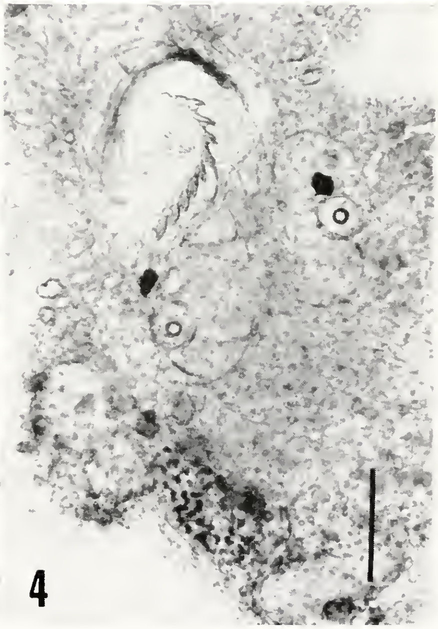
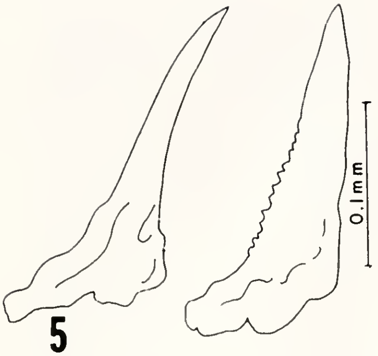


FIGURE 1. Adult *Coryphella stimpsoni* measuring 33 mm in length.

FIGURE 2. Juvenile *C. stimpsoni* measuring 6 mm in length.

FIGURE 3. Radula of a juvenile *C. stimpsoni* at 7 rachidian tooth stage. Scale equals 0.01 mm.

FIGURE 4. Juvenile at 8 rachidian tooth stage showing radula, eyes, cerebral ganglion and statocysts. Scale equals 0.03 mm.



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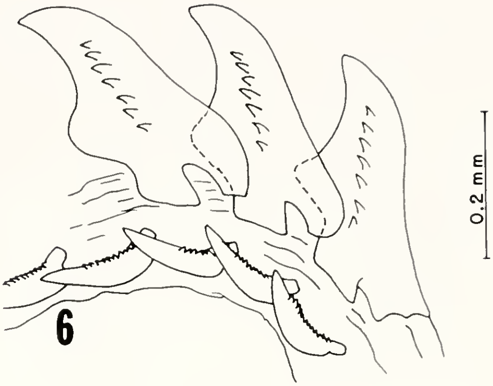

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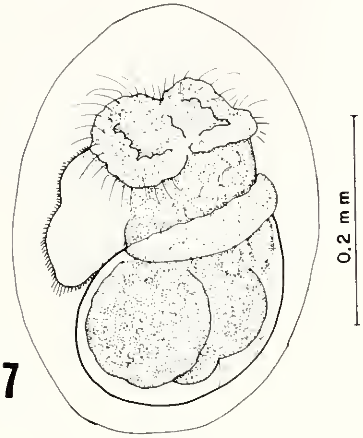

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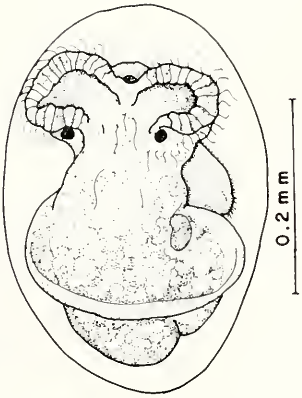

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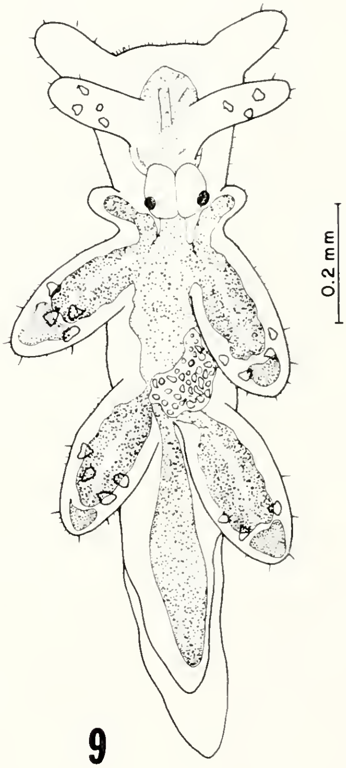

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FIGURE 5. Two lateral radular teeth of an adult *C. stimpsoni* showing variations of the shape and presence of denticles.

FIGURE 6. Portion of radular ribbon of *C. stimpsoni* showing the single row of rachidian teeth and laterals of one side.

strate in a probing manner and are utilized for holding the prey during feeding. The rhinophores are very contractile and can retract to one half their length upon being touched or when they come in contact with a foreign object such as a sea anemone. When contracted the rhinophores appear to be ringed but in extension during undisturbed activity this configuration is lacking. In the living forms the rhinophores are tinged a light orange and the distal third has a white external pigmentation.

The foot is broad, well developed with two short anterior lateral extensions which add $\frac{1}{5}$ to the total width of the foot at their point of origin. The width of the foot is relatively constant and the posterior end forms a rather abrupt point. During crawling activities the posterior portion of the foot does not project far from the body.

The cerata have a central core of the digestive gland which is red-brown in color. In each ceras this core terminates at the distal end just before an epidermal pigmentation of white dots appears. In some cerata these external dots are numerous and close together forming a ring and the tip beyond this ring is the translucent white color of the body. The cerata are round in cross section and taper evenly toward the distal end. In several specimens there were patches of two to five cerata which did not contain the central core of the digestive gland.

The reproductive opening, anus and excretory pore all open on the right side of the animal between the mantle edge and the foot. The anus is located $\frac{3}{4}$ posteriorly on the right side, the genital complex anteriorly on the same side and the smaller excretory pore in between nearer the genital openings. The area between foot margin and the first longitudinal row of cerata is pigmented with an epidermal white pigmentation of the same type found associated with the cerata and the rhinophores.

The triseriate radula is variable. In the fifteen radulae examined, the number of rows of teeth varied from 24–31. The denticulations on either side of the central tooth ranged from 8–13 and those of the lateral teeth varied from 9–11 fine denticles to no observable denticulations. On several radulae the younger lateral teeth of the ribbon appeared to be more slender and to have fewer denticles (Fig. 5).

The central rachidian teeth measured 0.28–0.38 mm in height (Fig. 6). The teeth are attached to the radular membrane by two square lateral projections which were described and figured by Krause (1892) and Knipowitsch (1902). The lateral teeth ranged between 0.17–0.22 mm in length and both slender and stout teeth as figured by Krause and Knipowitsch were found.

Two well-developed jaws are associated with the buccal mass. The masticating edges of the jaws form an oval ring lining the oral opening into the buccal mass. Bergh (1885) illustrated a portion of the masticating edge of the jaw showing the denticles which agrees with my observations. Under oil immersion the denticles are found to be covered with tubercles which are most prominent on the reflected

FIGURE 7. Side view of developing veliger of *C. stimpsoni* showing the mantle fold and shell.

FIGURE 8. Encapsulated veliger of *C. stimpsoni* with velum, foot, eyes, mantle fold and larval heart.

FIGURE 9. Juvenile *C. stimpsoni* with four primary cerata with differentiated cnidosacs. Note the beginning of the first pair of cerata in front of the anterior pair.

outer edges of the masticating portion and considerably worn down toward the oral opening into the buccal mass.

Feeding activity

When numbers of *C. stimpsoni* were observed in Maine, the animals were found crawling on an extensive mud flat. Screening of the substrate was undertaken to determine a possible food source for the nudibranchs. The burrowing anemones, *Edwardsia elegans* Verrill and *Halcampa duodecimcirrata* (Sars), were found in the same habitat and later in the laboratory when with these organisms, *C. stimpsoni* exhibited a definite feeding response. The feeding animal projected the outer lip and mouth forward and lowered the oral tentacles to hold the prey. The epidermis of the anemone was rasped away by the radula and ingested. Specimens collected in 1969 were maintained in the laboratory using the hydroid *Tubularia* sp. as their food organism.

Reproduction and development

Reproduction and development in *Coryphella stimpsoni* has been observed over a two-year period, and in each year the nudibranchs were collected in October, taken to the Marine Science Institute and held in the laboratory tanks. The animals copulated for several hours during which time the exchange of sperm occurred when the two individuals were aligned in opposite directions with their reproductive openings in communication. There was little movement during copulation.

Egg masses, when viewed from above, were normally deposited in concentric whorls starting from the center and moving counterclockwise. The coil thus produced varied from $1\frac{1}{2}$–$2\frac{1}{4}$ whorls and adhered tightly to the surface on which it was extruded. In one case the individual whorl measured 2 mm in width and the entire egg mass measured 10 mm in diameter. The individual uncleaved eggs measured 0.25 mm in diameter and the egg capsule surrounding the single egg measured 0.41 mm in length by 0.30 mm in width. The egg masses were deposited over a period of approximately 15–20 days during the month of December. The algal species *Ascophyllum nodosum* and *Chondrus crispus* were introduced to the tanks and utilized by the nudibranchs for egg deposition. Although both these species are present on the intertidal area where the nudibranchs were collected and may serve as a substrate for egg deposition, *C. stimpsoni* also deposited eggs on the sides of the tanks and the undeside of several rocks in the tanks.

Development time varied during the two years of observations and is influenced by temperature. The time of development from egg deposition until the juvenile crawled from the egg mass was 52 days at a temperature ranging from 4.0° C to 5.0° C. The time of development at a temperature ranging from 5.0° C to 8.5° C was from 25 to 34 days. This makes it difficult to predict development in nature and continual observations of development in the Maine habitat have not been made.

The eggs cleave equally to the four-cell stage and a quartet of micromeres are given off clockwise at the eight-cell stage. A rounded ball of small cells results from repeated cleavages. Soon after this a cap-shaped gastrula is formed with a ventral depression. The gastrula changes shape to a rounded form and the velum begins to differentiate. There is no observable internal differentiation, the embryo appears opaque due to the presence of a large amount of yolk and there is no movement within the egg capsule.

Differentiation of the foot and velum of the nudibranchs continues with the development of two velar lobes and cilia. The foot, without an operculum, enlarges on the ventral side. At this stage a large yolk mass of the body begins to differentiate into three lobes. These lobes are partially united anteriorly, but posteriorly the mass on the left side is single and there are two smaller lobes on the right side. The two right lobes are united to one another and then together with the larger left yolk mass. The larvae begin to show some movement within the egg capsules. Further differentiation occurs with the formation of the mantle fold, the secretion of a thin shell surrounding the body of the veliger, and the development of a pair of eyes and statocysts. The veligers move within the egg capsule but not at any great speed and in many cases, they remain still with the velar cilia continuing to move randomly. The shell is very thin and is present for only a short period of time (Figs. 7 and 8).

While still in the capsule larval characteristics begin to disappear and the veliger begins to resemble an adult. The mantle fold moves posteriorly forming the edge of the mantle. The four primordia of the cerata develop in front of the fold which subsequently becomes indistinct. However, it can still be seen just above the large digestive gland and posterior to the cerata primordia. The velum is reabsorbed and the velar cilia are reduced to patches near the eyes. As the velum is reabsorbed the cilia on the muscular foot become active. Although the entire surface of the foot is covered, the cilia are numerous and close together along the anterior margin. These cilia cause the rotation of the embryo which is very minor at this stage. Small rudiments of the oral tentacles and rhinophores appear on the head and the dorsal portion of the visceral mass shows differentiation of four digestive gland primordia which develop simultaneously, in most cases while the nudibranchs are still within the egg capsule.

The juveniles hatch from the egg mass from 25 to 52 days after egg deposition depending on the temperature of the surrounding water during development. Several days before hatching, the interior portion of the stroma surrounding the eggs becomes opaque and is invaded by microörganisms. Nematodes, protozoans and harpacticoid copepods can be seen moving through the coils. The outside of the egg string remains intact for about a week after the hatching process is complete. The individual egg capsules become slightly opaque just prior to their rupture and when the capsule wall collapses the animal crawls away from the flaccid structure. Eclosion is not clear. There is a great deal of activity of the foot prior to hatching and the cilia of the foot, especially the numerous anterior cilia, beat continually. Juveniles which escape from the egg capsule have been found, in some cases, to have from two to four radular teeth present which they may use to rasp through the capsule.

Juveniles crawl directly from the egg capsule and take up an immediate benthic existence. They were positively phototactic and gathered on the sides of the vessels nearest the window if food was not present. However, if food was present, they crawled toward the hydroid to feed. The newly hatched juveniles appear opaque white with large granules of yolk in the body. The body is well differentiated from the foot. The surface epithelium is entirely ciliated with greater concentrations of cilia on the anterior portion of the foot and around the mouth area. Stiff sensory bristles occur on the oral tentacles, rhinophore primordia and the four primary cerata. Statocysts and eyes are present and the small juveniles continue to move over the surface of the substrate by both muscular action of the foot and by ciliation of the foot. In an experimental group, mixed cultures of the algae *Dunaliella* sp., *Monochrysis lutheri* and *Isochrysis galbana* were introduced into the bowls. The algae could be seen rotating in the stomach and gave the digestive system a green coloration. However, the animals were not able to survive on this diet and it is questionable as to whether they were able to derive any nutrition from the algae.

The second time the life cycle was studied (1969–70), the hatched juveniles were immediately placed with the gymnoblast hydroid *Sarsia mirabilis* which was collected from the intertidal rocks at Nahant. Feeding began immediately and, in the continual presence of the food organism, individuals reached a length of 6.6 mm at 60 days post-hatch with the development of approximately 60 cerata on the dorsal surface (Fig. 2).

After ingestion there is an immediate coloration of the stomach as well as of the four extensions of the digestive gland in the four primary cerata. At this stage the left digestive gland supplies the anterior left and posterior pair of primary cerata. The smaller right digestive gland supplies the right anterior ceras. Externally the cerata appear bulky in feeding juveniles and at their tips the cnidosacs differentiate as internal opaque white structures. Squash preparations of living animals show an abundance of unexplored nematocysts stored in the cnidosacs. An animal that is disturbed with a probe under a dissecting microscope is seen to emit these unexploded nematocysts from the tip of the cerata. It would appear from the number of nematocysts present at any one time that they must be continually given off from the cerata in order to eliminate the structures from the digestive system. Food within the digestive gland extensions is continually flowing to and from the stomach by contractions of the cerata and ciliary action of the cells lining the gut. Very soon after feeding begins fecal pellets accumulate. The food-organism was orangish-pink but the fecal pellets were red. The nudibranchs graze on the entire hydroid colony but appear to prefer the undifferentiated tips of growing coenosarc.

The radula is used to graze on the hydroid and can be seen in a rasping motion during the feeding process. Squash preparations and whole mounts utilizing Turtox CMCS mounting media have shown that the newly hatched juveniles already have a radula with three to five rachidian teeth with well-developed denticles (Fig. 3). The radular teeth continue to increase in numbers during growth and in 10–40 day post-hatch juveniles there are from 8–12 rachidian teeth on the radular ribbon (Fig. 4). Toward the posterior portion of the radular ribbon (at about rachidian tooth 7) a pair of slender lateral teeth can be seen on the radular ribbon.

Juveniles that actively feed on the gymnoblasts begin to show formation of new cerata after about ten days of feeding. The first pair of new cerata form in front of the anterior pair of primary cerata (Fig. 9). The second pair arise in front of the second pair of primary cerata and the third single ceras differen-

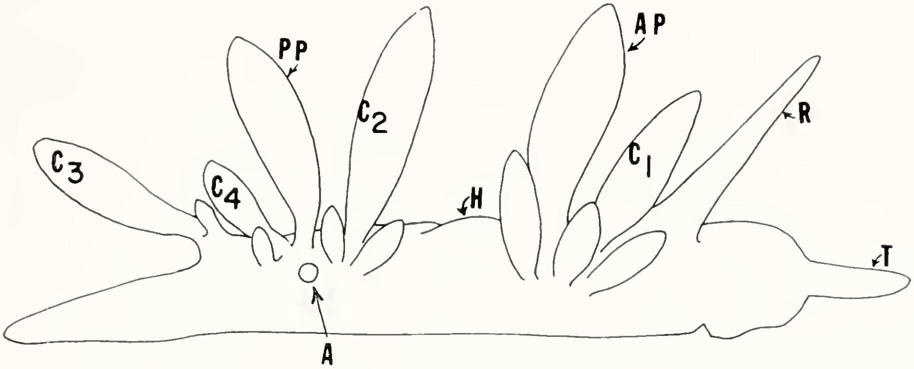

FIGURE 10. Diagrammatic representation of the sequential development of the cerata from the primary cerata (AP, anterior primary ceras; PP, posterior primary ceras). C_1, C_2 and C_4 represent the right cerata of pairs while C_3 is a single posterior ceras. Other structures shown include the oral tentacle (T), anal opening (A), heart bulge (H) and rhinophore (R).

tiates at the posterior extremity of the nudibranch body (Fig. 10). The foot extends beyond this portion of the body. The cnidosacs of these new cerata differentiate several days after the initial cerata formation. The fourth pair of cerata develop just behind the second pair of primary cerata. Cerata formation continues in pairs with a second lower group being established on either side of the middle two rows. At this stage the nudibranchs measure 2.0–4.5 mm in length. The eyes gradually sink to a position on either side of the cerebral ganglion and are relatively small at this later stage in comparison to earlier post-hatch stages. The cerata are not as bulky in the 4.5 mm length nudibranchs and the whole animal takes on a slender adult-like appearance. There are scattered spots of white epidermal pigmentation at the four-cerata stage and as the animal continues to differentiate they coalesce into rings around the cerata (Fig. 2). The adult heart is clearly visible and beating at a length of 4.6 mm (Fig. 11).

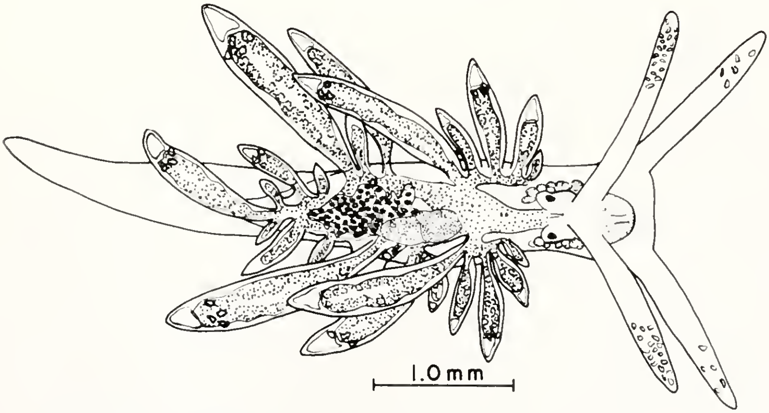

FIGURE. 11. Juvenile *Coryphella stimpsoni* showing development of cerata at a length of 4.6 mm.

DISCUSSION

Verrill (1880) indicated that *Coryphella stimpsoni* bore a close resemblance to *Coryphella salmonacea* Couthouy. Both species have evenly spaced cerata as opposed to those species of *Coryphella* with cerata in distinct groups. In addition both species show a similar type of development in that the young crawl directly from the egg mass when hatching (Morse 1969a, 1969b). The differences between adults of the two species are that *C. stimpsoni* has a distinct trefoil surrounding the mouth, deposits egg strings in December, usually occurs on mudflats and will feed on burrowing sea anemones, while *Coryphella salmonacea* is generally larger, has only lateral bulges surrounding the circular mouth, deposits egg masses in March and April, and has only been found on rocky shores.

The radula of *C. stimpsoni* is variable within one animal as well as among several nudibranchs. Variations include the dentition of the central and lateral teeth, shape of the lateral teeth and numbers of rows of teeth in the adults. This is in contrast to Odhner's (1939) opinion that in the genus *Coryphella*, the radula is relatively constant in each species. Schönenberger (1969) described the variation of the radula of *Trinchesia granosa* Schmekel throughout its life-history. A comparable variation was seen in *C. stimpsoni* and points out the need for further studies on large numbers of nudibranchs of a species to determine if variability exists.

The development pattern of *Coryphella stimpsoni* is similar to development type 3 as described by Thompson (1967); that is the animal begins benthic life immediately after breaking out of the egg membrane. Two reports of development type 3 in eolid molluscs were listed by Thompson (1967), *i.e.*, the report of Tardy (1962) on *Eolidina alderi* and that of Roginskaya (1962a) on *Cuthona pustulata*. In a second paper, Roginskaya (1962b) reported the development of *C. pustulata* in more detail. Schönenberger (1969) reported a similar development in *Trinchesia granosa*. The term direct development implies a juvenile crawling from the egg mass to a benthic existence, however *E. alderi* crawls out with the veliger shell still attached and *C. pustulata* loses the veliger shell after breaking out of the capsule but while still in the stroma of the egg mass. Both *T. granosa* and *C. stimpsoni* crawl out of the egg mass as juveniles with no shell.

Rao (1961) described the differentiation of the eolid *Cuthona adyarensis* which assumed a benthic existence after a short free-swimming veliger phase. Rao (1961), Roginskaya (1962b) and Schönenberger (1969) all reported in detail the development of the oral tentacles, the rhinophores and the sequence of differentiation of the cerata. It appears that these processes are very similar although there is a noticeable difference in the sequence of cerata differentiation which may prove to be species specific. In *Coryphella stimpsoni* the oral tentacle rudiments, rhinophore rudiments and the large dorsal visceral mass are already present when the nudibranch breaks through the membrane. The visceral mass soon equally forms four primordia of the primary cerata. The yolk present in the juvenile is enough to nourish the juveniles during the period of differentiation, although without the natural food further development is halted.

Thompson (1967) noted that four species of nudibranchs reported to have development type 3 have a boreo-arctic distribution which is also true of *Coryphella stimpsoni*. One of the four species mentioned by Thompson, *Cadlina laevis*, has been collected by the author within two miles of the locality from which the *Coryphella stimpsoni* were taken. *C. stimpsoni* is similar to *Cadlina laevis* in that the veligers and hatching juveniles are filled with yolk granules. In both species the embryonic period is long, there is a reduction of the velum and shell, and there is an absence of an operculum.

The term direct development is a bit misleading in the literature because of prior use, especially in relation to insect development. In the latter it generally refers to a series of embryonic stages where the animal's form closely resembles the adult. The juveniles do not have a distinct set of "larval characteristics." Among the molluscs the term has only been used for nudibranchs although many prosobranchs and pulmonates show a similar pattern of development. The important factors remain that there are a group of opisthobranchs which crawl directly from the enveloping capsule without a free-swimming veliger stage and in most cases when crawling-out show no external evidence of typical opisthobranch veliger morphology. Thompson (1967) has listed the characteristic reduction of veliger structures during early development which holds true of *C. stimpsoni* and which appears common to this form of development. It is suggested that his classification, *i.e.*,—"Development Type 3" be utilized in place of the often misleading term "direct development."

SUMMARY

1. The morphology and life history of the nudibranch mollusc, *Coryphella stimpsoni* (Verrill 1879) is described.

2. Adults were collected in October and deposited egg strings in December.

3. Development corresponds to Thompson's (1967) Type 3, *i.e.*, juveniles crawl directly from the egg mass and begin benthic life.

4. Development time from egg deposition to the benthic crawling stage was from 25 to 52 days.

5. Veligers are characterized by a large amount of yolk, a reduced velum, a thin shell and no operculum.

6. Sequential development of tentacles, rhinophores and cerata and the differentiation of other organs such as cnidosacs, heart and digestive structures in the juveniles are described.

7. This is the first report of an eolid nudibranch from New England which crawls directly from the egg mass as a juvenile to begin benthic life.

LITERATURE CITED

BERGH, R., 1885. Beiträge zur Kenntniss der Aeolidiaden VIII. *Verh. Zool. Bot. Ges. Wein*, **35**: 1–60.

KNIPOWITSCH, N., 1902. Zoologische Ergebnisse der Russischen Expeditionen nach Spitzbergen. *Ann. Mús. Zool.*, **7**: 355–459.

KRAUSE, A., 1892. Mollusken von Ostspitzbergen. *Zool. Jahrb. Abt. Syst. Oekol. Geogr. Tierre*, **6**: 339–376.

MORSE, M. PATRICIA, 1969a. Contribution to the knowledge of New England Nudibranchs. *Ann. Rep. Amer. Malacol. Union*, **1969**: 7–18.

MORSE, M. PATRICIA, 1969b. Direct development in the nudibranch mollusc, *Coryphella stimpsoni* Verrill 1880. *Amer. Zool.*, **9**(3): 618.

ODHNER, NILS HJ, 1939. Opisthobranchiate mollusca from the Western and Northern coasts of Norway. *Det. Kgl. Norske Vidensk. Selsk. Skr.* **1**: 1–92.

RAO, K. V., 1961. Development and life history of a nudibranchiate gastropod *Cuthona adyarensis* Rao. *J. Mar. Biol. Ass. India,* **3**: 186–197.

ROGINSKAYA, I. S., 1962a. The egg masses of nudibranch molluscs of the White Sea. Biologiya Beloga Morya, *Trudy Belomorskoy Biologisheskoy Stantsii Morsk. Gosundarst Univ.*, **1**: 201–214 (In Russian).

ROGINSKAYA, I. S., 1962b. Biology, reproduction and development of *Cuthona pustulata* (Gastropoda, Nudibranchia). *Dokl. Akad. Nauk SSSR,* **146**(2): 488–491 (In Russian).

SCHÖNENBERGER, NORBERT, 1969. Beiträge zur Entwicklung und Morphologie von *Trinchesia granosa* Schmekel (Gastropoda, Opisthobranchia). *Pubbl. Sta. Zool. Napoli,* **37**: 236–292.

TARDY, J., 1962. Cycle biologique et métamorphose d'*Eolidina alderi* (Gastéropode, Nudibranche). *C. R. Hebd. Seances Acad. Sci. Paris,* **225**: 3250–3252.

THOMPSON, T. E., 1967. Direct development in a nudibranch, *Cadlina laevis,* with a discussion of developmental processes in opisthobranchia. *J. Mar. Biol. Ass. U. K.,* **47**: 1–22.

VERRILL, A. E., 1879. Notice of recent additions to the marine fauna of the eastern coast of North America. *Amer. J. Sci.,* **17**: 309–315.

VERRILL, A. E., 1880. Notice of recent additions to the marine invertebrata of the northeastern coast of America, with descriptions of new genera and species and crucial remarks on others. *Proc. U. S. Nat. Mus.,* **3**: 356–405.

Reference: *Biol. Bull.*, **140**: 95–103. (February, 1971)

JUVENILE NEMATODES (*ECHINOCEPHALUS PSEUDOUNCINATUS*) IN THE GONADS OF SEA URCHINS (*CENTROSTEPHANUS CORONATUS*) AND THEIR EFFECT ON HOST GAMETOGENESIS

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Although sea urchins have been used as biological research material for many years, particularly as a source of gametes, there have been few reports of infection of these animals by nematodes. The nematode *Echinomermella grayi* (Gemmill and von Linstow, 1902) tentatively placed in the Mermithoidea by Chitwood (1933), is known from a single specimen found in the perivisceral coelom of *Echinus esculentus* off Britain, and perhaps a second specimen (Irving, 1910; Ritchie, 1910). Juvenile specimens of gnathostomatid nematodes have been reported twice from sea urchins: *Echinocephalus uncinatus* "perhaps accidentally" in tropical sea urchins (Shipley and Hornell, 1904) and a single specimen of *E. pseudouncinatus* in the gonads of *Arbacia punctulata* at Woods Hole, Massachusetts (Hopkins, 1935; Millemann, 1951). Nematodes have also been found in the gonads of the urchin *Astropyga pulvinata* off Acapulco, Mexico, but these have not been identified (J. S. Pearse, unpublished observations). Neither Hyman (1951, 1955), Johnson (1968), Johnson and Chapman (1969), nor Holland and Holland (1970) cite any other record of nematodes in echinoids.

We report herein the regular occurrence of juvenile *Echinocephalus pseudouncinatus* in the gonads of *Centrostephanus coronatus* off Southern California. Between about 40 and 80% of the sea urchins contained the nematodes in their gonads. Moreover, gametogenesis in parts of the gonads was profoundly affected by the presence of the nematodes.

Materials and Methods

Most specimens of *Centrostephanus coronatus* were collected from 3 to 10 m depth by scuba diving off the northeastern shore of Big Fisherman's Cove, Santa Catalina Island, near the Santa Catalina Marine Biological Laboratory. Other samples were taken from Pin Rock in Catalina Harbor on the opposite side of Santa Catalina Island, and from Whistler's Reef off the mainland coast of California near Corona del Mar. These three areas are all in quite different waters: Big Fisherman's Cove is on the Santa Catalina Channel side of Santa Catalina Island; Catalina Harbor, although less than 3 km distant, is on the Pacific side of the island; and Whistler's Reef is about 25 km away on the opposite side of Santa Catalina Channel. Moreover, one sample of gonads from *C. coronatus* was taken on 26 November 1968 from Bahia Tortola (Turtle Bay) on the west-central coast of Baja California, Mexico.

The sea urchins were lodged deep in crevices among the rocks during the day and were extracted with the aid of a bent metal rod. During the night, animals foraged in the open and were more easily collected.

The specimens were dissected within a day after collection and all the gonads were carefully inspected for the presence of nematodes. Records were kept of the presence or absence of worms in the gonads, with notation of their relative abundance.

Gonads with nematodes were fixed in Bouin's solution or warm formalin-alcohol-acetic acid-water solution (FAA; 1:5:1:4). Some of the fixed specimens were dehydrated in an acetone series, cleared in benzene, embedded in paraffin, sectioned at 10 μ, and stained in hematoxylin and eosin. Others were dissected whole from the gonads.

TABLE I

Incidence of specimens of Centrostephanus *infected with* Echinocephalus

Date	Number of *Centrostephanus*	Number (and per cent) infected with *Echinocephalus*
	Big Fisherman's Cove, Santa Catalina Island	
11 Aug. 69	38	26 (68.5)
20 Aug. 69	7	5 (71.5)
26 Aug. 69	18	14 (78.0)
31 Aug. 69	17	14 (82.5)
15 Oct. 69	10	9 (90.0)
29 Nov. 69	20	16 (80.0)
2 Feb. 70	18	13 (65.0)
9 Mar. 70	25	21 (80.0)
	Catalina Harbor, Santa Catalina Island	
28 Aug. 69	22	9 (41.0)
	Whistler's Reef, Corona del Mar	
10 Nov. 69	20	8 (40.0)
20 Nov. 69	18	7 (39.0)

OBSERVATIONS

Frequency of nematode infection in Centrostephanus coronatus

A total of 153 specimens of *C. coronatus* were collected from Big Fisherman's Cove, Santa Catalina Island, and examined for the presence of nematodes in their gonads. Of these, 118 (78%) were infected (Table I). The percentage infected in each sample ranged from 65 to 90%. All of the urchins in the August samples were sexed by microscopic examination of gonadal smears; there were 46 males and 34 females, and 31 and 28 were infected, respectively.

All the samples of *C. coronatus* from both Pin Rock in Catalina Harbor and Whistler's Reef off Corona del Mar had lower incidences of infection than those from Big Fisherman's Cove (Table I). Moreover, not only were a smaller percentage of animals infected, but those that were contained fewer nematodes. Most of the infected urchins in Big Fisherman's Cove had multiple infections, with several nematodes in each of the five gonads. In contrast, most of the infected urchins from both Pin Rock and Whistler's Reef had only one or two nematodes in only one or two of the gonads, and detection of the infection required careful dissection of all the gonads.

Seventeen specimens of *C. coronatus* were also collected and dissected on 26 November 1968 from Bahia Tortola (Turtle Bay) on the west-central coast of Baja California. At the time of the collection, the presence of nematodes was not known, but during the dissection, one gonad was found with an encysted nematode.

The juvenile nematode

The nematodes we found in the gonads of *C. coronatus* are all juveniles. They do not differ in any respect from Millemann's (1951) description of *Echinocephalus pseudouncinatus*. The most significant feature for identification is the structure of the head bulb (Fig. 1A), which bears 6 rows of about 40 hooks each, with a lateral separation of the hooks and smaller hooks on the dorsal and ventral areas. Figure 1B shows the tail, which was not drawn by Millemann (1951).

Echinocephalus pseudouncinatus was originally described from numerous juvenile specimens found in the foot of pink abalones *Haliotus corrugata* from San Clemente Island off Southern California (Millemann, 1951). This is the only species of *Echinocephalus* presently known from Southern California. Juveniles of another species of *Echinocephalus, E. uncinatus,* are known to infect molluscs elsewhere (Shipley and Hornell, 1904; Baylis and Lane, 1920), and it is likely that *E. pseudouncinatus* juveniles regularly infect both molluscs and *C. coronatus* in Southern California. Among the sea urchins of Southern California, however, juvenile *E. pseudouncinatus* seem to infect *C. coronatus* specifically. Hundreds of specimens of the sea urchins *Strongylocentrotus purpuratus, S. franciscanus,* and *Lytechinus anamesus* from Southern California have been dissected during the past year and only a single specimen of *E. pseudouncinatus* has been found in the gonad of one *S. purpuratus* collected near San Diego. The single, remarkable record of what seems to be a juvenile specimen of *E. pseudouncinatus* in the gonad of a specimen of *Arbacia punctulata* at Woods Hole, Massachusetts, should again be noted (Hopkins, 1935; Millemann, 1951).

Possible adult hosts

Adults of the genus *Echinocephalus* inhabit the intestine, usually the spiral valve, of elasmobranchs (Hyman, 1951; Yamaguti, 1961). The most conspicuous elasomobranch in the vicinity of Big Fisherman's Cove, Santa Catalina Island, is the California horned shark *Heterodontus francisci.* This species is known to feed on sea urchins and its teeth and bones often are colored purple, presumably from an accumulation of sea urchin naphthoquinone pigments (Leighton Taylor, Scripps Institution of Oceanography, personal communication). A similar purple coloration of teeth and bones occurs in the sea otter *Enhydra lutris,* which feeds on sea urchins (Fox, 1953). Other species of horned sharks are known to feed on sea urchins, including *H. phillipi* and *H. galeatus* off southeast Australia, and *H. japonicus* off Japan (Smith, 1942). Moreover, Saville-Kent (1897, page 192) noted the teeth of *H. phillipi* ". . . are not infrequently stained a deep purple, through constant indulgence in a dietary of the commoner purple urchin."

Accordingly, three specimens of California horned sharks were collected in February 1970 from Big Fisherman's Cove, Santa Catalina Island, and the intestinal contents examined. One large female, measuring 76 cm total length,

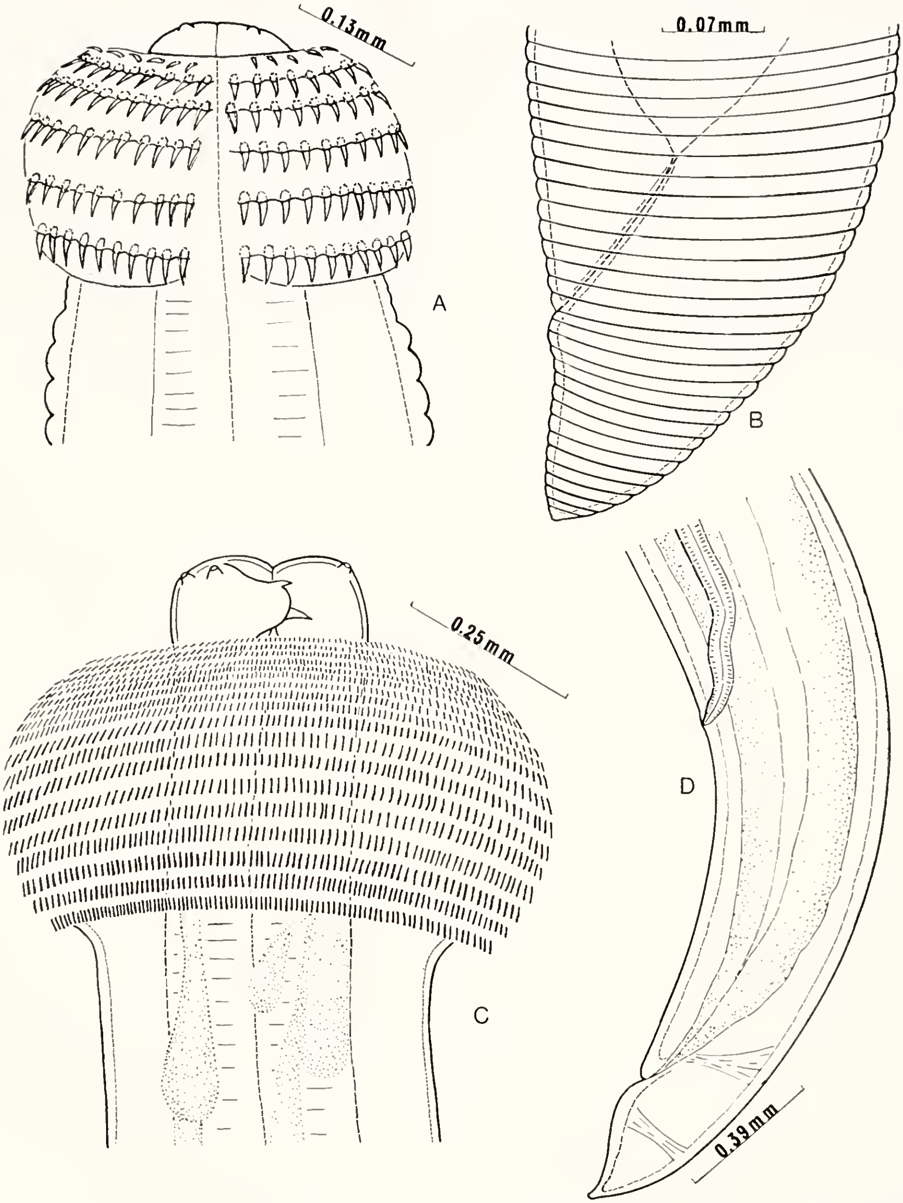


FIGURE 1. Juvenile head, lateral view (A) and juvenile tail (B) of *Echinocephalus pseudouncinatus* from the gonad of *Centrostephanus coronatus*, and female head, lateral view (C) and female tail (D) of *Echinocephalus* sp. from the spiral valve of *Heterodontus francisci*.

contained three adult specimens of *Echinocephalus* in its spiral valve. The adult worms, however, have 18 rows of hooks on the head bulbs, each containing 150–200 hooks and therefore, by current taxonomic criteria, cannot be considered the same species as the juveniles in the sea urchin. The number and arrangement of hooks on the head bulb is the same as in *E. southwelli* Baylis and Lane, 1920, but the vulva and cervical sacs are much more anterior than in that species. Since we found no males, no attempt is made to describe the nematode as a new species, but the measurements, a brief description, and drawings (Fig. 1C, D) are given in order that parasitologists might be aware of this previously unknown host-parasite relationship.

Young entire female: Length = 22.46 mm; maximum body diameter = 0.68 mm; esophagus = 3.48 mm; esophagus-vulva = 17.42 mm; vulva-anus = 1.19 mm; tail = 0.37 mm; anal body diameter = 0.26 mm; head bulb = 0.75 × 0.44 mm; lip region = 0.26 × 0.12 mm.

Anterior of one female: Length = 17.42 mm; maximum body diameter = 1.2 mm; head bulb = 0.93 × 0.17 mm; lip region = 0.44 × 0.26 mm.

Posterior of two females: Length = 19.86–24.0 mm; maximum body diameter = 1.34 mm; vulva-anus = 2.75–3.67 mm; tail = 0.58–0.70 mm; anal body diameter = 0.16 mm.

Description: Cuticle sometimes coarsely but irregularly annulated, probably due to contraction, each annulus bearing finer striations. Eighteen rows of hooks on the head bulb, composed of 150–200 hooks each, larger hooks toward posterior; distance between rows less than length of hooks; maximum length of hooks 0.035 mm. Two large lips, each bearing two prominent papillae; overlapping lobe of lip bears two toothlike projections. Cervical sacs extending about $\frac{1}{2}$ length of esophagus in young female. Deirids not observed. Tail broadly conical, narrowly rounded at tip. These adult nematodes, as well as a representative collection of juvenile *E. pseudouncinatus* from *C. coronatus*, are held in the Nematode Collection of the University of California, Davis.

Nematode effect on urchin gonads

The echinoid gonad consists of five primary tubules that grow orally along the interambulacrals from each of the aborally situated gonopores (Fig. 2). The five primary tubules branch repeatedly so that in adults the five gonads are large organs of intertwining tubules. In *C. coronatus* the gonads are anchor-shaped, with the "flukes" of the anchor situated orally. The gonads are enveloped completely with the thin, ciliated epithelium of the perivisceral coelom, and strands of this epithelium secure the gonad to the inner surface of the test wall and to the gut. Under the perivisceral coelomic epithelium, thin layers of smooth muscle and connective tissue cover the inner germinal epithelium. The germinal epithelium is derived from the hemal strand and consists of both reproductive cells (gonials, spermatocytes or oocytes, spermatozoa or ova) and accessory cells (nutritive phagocytes). Often there is a space ("perihemal coelom") between the perivisceral coelomic epithelium and the muscle layer and another space ("hemal sinus") between the muscle layer and the germinal epithelium.

Young juvenile nematodes were lodged between the perivisceral coelomic epithelium and the germinal epithelium; that is, within the hemal or perihemal

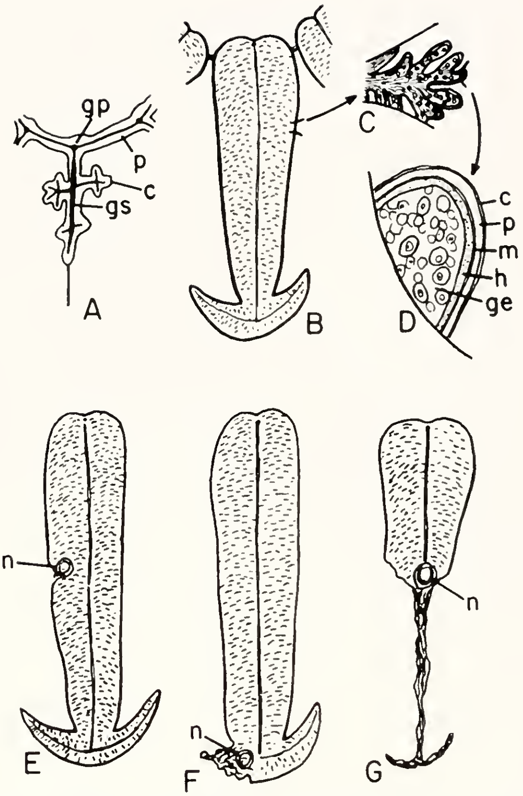
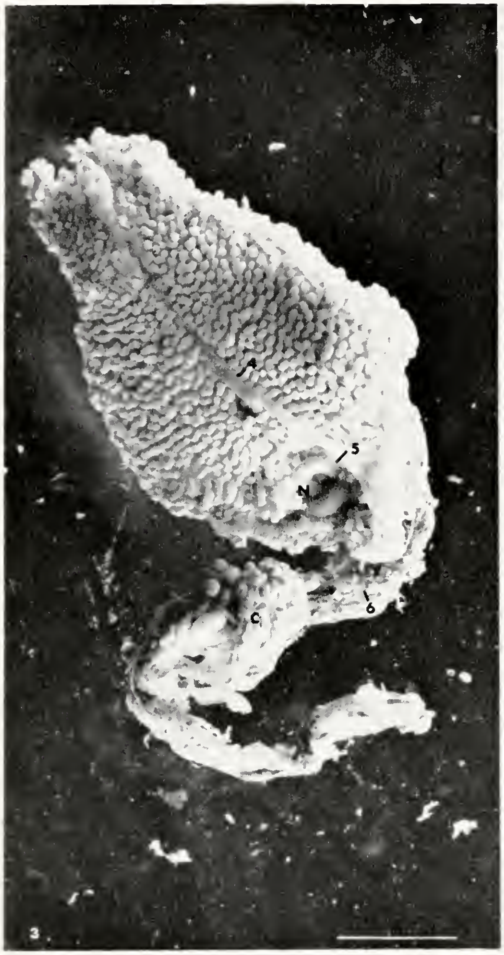
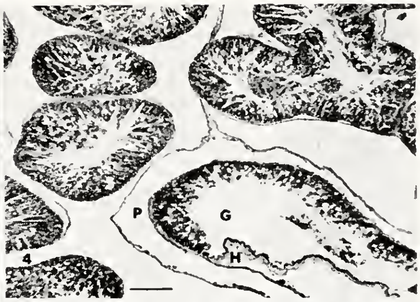


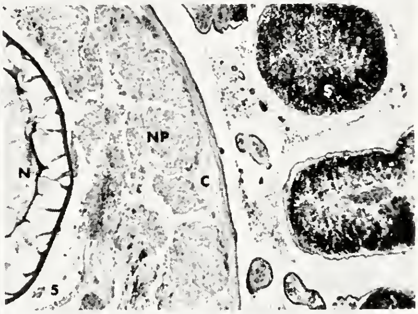
FIGURE 2. Diagrams showing the structure of sea urchin gonads during early development in a juvenile urchin (A) (after Tahara and Okada, 1968), when fully developed in an adult *Centrostephanus coronatus* (B, C, D), and the effect on the gonad when infected by juvenile *Echinocephalus pseudouncinatus* encysted near the end of a peripheral tubule (E), in the main tubule leading to one of the flukes of the gonad (F), and in the main tubule (gonoduct) near the middle of the gonad (G). All of the gonads are shown from their perivisceral coelomic sides with the oral portion down; abbrev.: c, perivisceral coelomic epithelium; ge, germinal epithelium; gp, position of gonopore; gs, hemal strand; h, "hemal sinus"; m, muscle; n, encysted nematode; p, "perihemal coelom."

spaces of the gonadal wall. Larger worms displaced most of the germinal epithelium in the infected tubule and thick, fibrous connective tissue encysted both the worm and associated germinal epithelium. Cysts with large juveniles protruded into the perivisceral coelom; large juveniles, probably nearly full-grown, broke through the wall of the gonadal cyst and extended free into the coelom.

Although gonadal tubules adjacent to those with the worms usually contained well-developed spermatogenic or oogenic cells, most of the tissue within the cysts in the infected tubules consisted of nongametogenic accessory or connective cells. Moreover, gametogenesis within infected tubules distal (oral) to the worm also seemed suppressed. When the infection occurred near the end of a side tubule, as was usually the case, there was little effect on the overall gametogenic state of the gonad (Fig. 2E). Occasionally, however, infection occurred within a main tubule and, in such cases, the gonads were shriveled distal to the sites of infection (Fig. 2F, G). In one dramatic example, two worms had lodged in the main tubule (probably the gonoduct) in the middle of one testis; spermatogenesis appeared







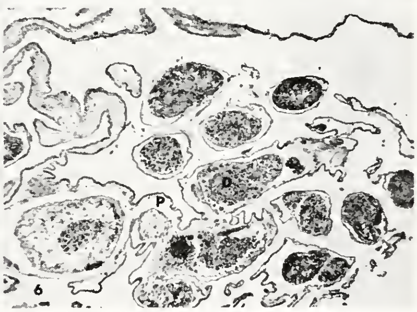


FIGURE 3. A testis of *Centrostephanus coronatus* infected with *Echinocephalus pseudouncinatus* showing a cyst containing one nematode (C) and a second nematode broken partially free of its cyst (N). The oral portion of the gonad is at the bottom of the photograph; note the normal-appearing aboral half of the gonad and the shriveled oral portion. Numbers 4, 5, and 6 correspond to Figures 4 through 6; the scale line indicates 5 mm.

FIGURE 4. Section through the normal-appearing aboral portion of the testis in Figure 3, showing the gonoduct (G) surrounded by perithemal (P) and hemal (H) spaces, and the testicular tubules full of spermatogenic cells (S).

FIGURE 5. Section through the nematode in the testis in Figure 3, showing a portion of the nematode (N) and extensive nutritive phagocytic tissue (NP) in a tubule surrounded by a fibrous cyst wall (C), and adjacent tubules full of spermatogenic cells (S).

FIGURE 6. Section through the shriveled oral portion of the testis in Figure 3 just below the nematode, showing the germinal epithelium full of degenerating cells (D) and large perihemal spaces (P). Figures 4–6 are from 10 μ paraffin sections stained with hematoxylin and eosin; all are at the same magnification and the scale line in Figure 4 indicates 100 μ.

active and normal in the aboral half of the testis, while the tubules were shrunken and filled with degradation products orally (Figs. 3–6).

The finding that encysted juvenile worms can suppress urchin gametogenesis has important implications regarding gametogenic control. Suppression does not seem to be due to the release and diffusion of some substance by the worms because gametogenesis is suppressed only oral to the infections rather than all around them. Moreover, the worms are coiled and encased in cysts produced by the urchin gonads; they are not likely to move through the gonads and disrupt gametogenesis. Rather, the encysted worms seem to block the passage of some material that is essential for gametogenesis. Such a gametogenic regulating substance, although never directly demonstrated, is almost certainly present because gametogenesis occurs in synchrony among all five gonads in individual sea urchins (Pearse, 1969). The encysted nematodes probably do not block transport of simple nutrients; radioactive tracing studies have shown that nutrient transfer in urchins occurs mainly through the perivisceral coelom (Farmanfarmaian and Phillips, 1962). Instead, the apparent blockage of gametogenesis by the juvenile nematodes indicates the presence of a hormonal substance which regulates gametogenesis in sea urchins and is transported *within* the gonad (perhaps through the perihemal or hemal spaces) rather than through the perivisceral coelomic fluid.

We are indebted to Dr. Russel L. Zimmer, Resident Director, for providing aid and facilities for these studies at the Santa Catalina Marine Biological Laboratory, Santa Catalina Island, and to Dr. Nicholas D. Holland, Dr. W. Duane Hope, Dr. Phyllis T. Johnson, and Mr. Leighton Taylor for advice and criticism. This study was supported in part by the Federal Water Quality Administration Grant No. 18050 DNV. The sample from Bahia Tortola, Baja California, was taken during Stanford Oceanographic Expedition, TE VEGA Cruise 20, supported by the National Science Foundation Grant Nos. GB6870 and GB6871. Part of the manuscript was prepared while the senior author was a visting faculty member of the University of California, Santa Cruz.

SUMMARY AND CONCLUSIONS

1. The juvenile phase of the nematode *Echinocephalus pseudouncinatus* occurs commonly in the gonads of the sea urchin *Centrostephanus coronatus* off Southern California. The gonads of about 78% of the urchins in Big Fisherman's Cove, Santa Catalina Island, were heavily infected with the juveniles. About 40% of the urchins at Pin Rock in Catalina Harbor, Santa Catalina Island, and at Whistler's Reef off Corona del Mar on the mainland coast of California were infected, although the infection was usually not as severe as in Big Fisherman's Cove. A juvenile, probably of *E. psuedouncinatus*, also was found in gonads of *C. coronatus* collected from Bahia Tortola off west-central Baja California. Although juveniles of *E. pseudouncinatus* also occur commonly in the foot of pink abalones, they only rarely infect other species of sea urchins in Southern California.

2. The California horned shark *Heterodontus francisci* seems a likely host of the adult phase of *E. pseudouncinatus*. However, when specimens of the horned

shark were examined, adult specimens of *Echinocephalus* were found which do not seem to be the same species as our juvenile specimens.

3. The juvenile nematodes were encysted mainly in the spaces (perihemal or hemal) between the perivisceral coelomic epithelium and the germinal epithelium of the *Centrostephanus* gonad. Large juveniles filled the host gonadal tubule and bulged into the perivisceral coelom. Host gametogenesis was suppressed in the infected gonadal tubule, especially in the oral parts of such tubules. Gametogenesis in adjacent tubules did not seem affected. When the juveniles were in major tubules of the host gonad, severe suppression of host gametogenesis occurred in the oral parts of the gonad. It is suggested that encysted juveniles block the passage through the gonadal tubules of some hormonal substance that regulates urchin gametogenesis.

LITERATURE CITED

Baylis, H. A., and C. Lane, 1920. A revision of the nematode family Gnathostomidae. *Proc. Zool. Soc. London*, **1920**: 245–310.

Chitwood, B. G., 1933. The systematic position of *Echinonema grayi* Gemmill, 1901. *J. Parasitol.*, **20**: 104.

Farmanfarmaian, A., and J. H. Phillips, 1962. Digestion, storage, and translocation of nutrients in the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.*, **123**: 105–120.

Fox, D. L., 1953. *Animal Biochromes.* Cambridge University Press, Cambridge, 379 pp.

Gemmill, J. F., and O. von Linstow, 1902. *Icthyonema grayi. Arch. Naturgesch.*, **68**: 113–118.

Holland, N. D., and L. Z. Holland, 1970. A bibliography of echinoderm biology, continuing Hyman's 1955 bibliography through 1965. *Pubbl. Staz. Zool. Napoli*, **37**: in press.

Hopkins, S. H., 1935. A larval *Echinocephalus* in a sea urchin. *J. Parasitol.*, **21**: 314–315.

Hyman, L. H., 1951. *The Invertebrates. Volume III. Acanthocephela, Aschelminthes and Entroprocta.* McGraw-Hill Book Co., New York, 572 pp.

Hyman, L. H., 1955. *The Invertebrates. Volume IV. Echinodermata.* McGraw-Hill Book Co., New York, 763 pp.

Irving, J., 1910. Nemertine within test of sea-urchin. *The Naturalist* (*London*), **1910**: 6.

Johnson, P. T., 1968. *An Annotated Bibliography of Pathology in Invertebrates other than Insects.* Burgess Publishing Co., Minneapolis, Minnesota, 322 pp.

Johnson, P. T., and F. A. Chapman, 1969. *An annotated bibliography of pathology in invertebrates other than insects supplement.* Misc. Publ. No. 1, Center for Pathobiology, University of California, Irvine, 76 pp.

Millemann, R. E., 1951. *Echinocephalus pseudouncinatus* n. sp., a nematode parasite of the abalone. *J. Parasitol.*, **37**: 435–439.

Pearse, J. S., 1969. Reproductive periodicities of Indo-Pacific invertebrates in the Gulf of Suez. I. The echinoids *Prionocidaris baculosa* (Lamarck) and *Lovenia elongata* (Gray). *Bull. Mar. Sci.*, **19**: 323–350.

Ritchie, J., 1910. Worm parasitic in sea-urchin. *The Naturalist* (*London*), **1910**: 94.

Saville-Kent, W., 1897. *The Naturalist in Australia.* Chapman and Hall, London, 302 pp.

Shipley, A. E., and J. Hornell, 1904. The parasites of the pearl oyster. *Rept. Govt. Ceylon Pearl Oyster Fish. Gulf Manaar* (Herdmann, London), **2**: 77–106.

Smith, B. G., 1942. The heterodontid sharks, their natural history and external development of *Heterodontus* (*Cestracion*) *japonicus* based on notes and drawings by Basford Dean. Pages 647–770 in E. W. Gudger, Ed., *The Basford Dean Memorial Volume, Archaic Fishes, Part II, Article VIII*, American Museum of Natural History, New York.

Tahara, Y., and M. Okada, 1968. Normal development of secondary sexual characters in the sea urchin, *Echinometra mathaei. Publ. Seto Mar. Biol. Lab.*, **16**: 41–50.

Yamaguti, S., 1961. *Systema Helminthum. Volume III. The Nematodes of Vertebrates. Parts 1 and 2.* Interscience Publishers, New York, 1261 pp.

Reference: *Biol. Bull.,* **140**: 104–116. (February, 1971)

LARVAL DEVELOPMENT OF *PAGURUS LONGICARPUS* SAY REARED IN THE LABORATORY. II. EFFECTS OF REDUCED SALINITY ON LARVAL DEVELOPMENT[1]

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Temperature and salinity define a set of conditions within which planktonic organisms can survive and develop. Thorson (1946) described the restriction of some meroplankters to Kattegat water in the Oresund which was presumed to be based on either a temperature, or a salinity discontinuity or both. Bary (1963a, b, c), in an extensive study of North Atlantic plankton, clearly demonstrated a relationship between zooplankton distribution and temperature-salinity distribution. Banse (1956) observed the distribution of polychaete and echinoderm larvae with respect to various water masses in Kiel Bay. He concluded that these larvae were restricted to their "Gebirtswasser" by the temperature-salinity characteristics of these water masses. In a subsequent paper (1959) he described a similar situation for copepods.

Survival and rate of development of decapod larvae are temperature-dependent phenomena. It has been shown for a variety of species that there is some optimal temperature range above and below which larval mortality increases (Boyd and Johnson, 1963; Chamberlain, 1961, 1962; Costlow, 1967; Costlow and Bookhout, 1962, 1968; Costlow, Bookhout and Monroe, 1960, 1962, 1966; Coffin, 1958, 1960). There is a unique range of temperature permitting survival for each decapod species so far studied. Further, it has been demonstrated that intermolt duration decreases with increasing temperature.

While temperature affects animal distributions both in the open sea and coastal waters, salinity has the greatest influence in coastal waters and estuaries. The ability of larvae to survive reduced salinity is therefore of extreme interest in the case of estuarine species.

Considerable research has been conducted to elucidate the effects of salinity on larvae of estuarine brachyurans (Chamberlain, 1961, 1962; Costlow, 1967; Costlow and Bookhout, 1962, 1968; Costlow *et al.,* 1960, 1962, 1966). Salinity strongly affects survival with tolerance ranges unique for each developmental stage and each species. There seems to be little effect on intermolt duration until the lethal salinity is neared, at which point a slight increase can be detected. Information is available for only three anomuran species, *Pisidia longicornis* (Lance, 1964),

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Pagurus samuelis (Coffin, 1958) and *Pagurus bernhardus* (Bookhout, 1964). The general trends are the same as for brachyurans.

In the present study the effects of reduced salinity on embryos and larvae of *Pagurus longicarpus* were determined. Efforts were made to detect changes in salinity tolerance with increasing developmental age.

Despite the importance of temperature in larval development, this parameter was not included as a variable in these studies. Limitations of equipment and time made it impractical to conduct experiments at several temperatures. While there may well be important interaction effects of salinity and temperature, it did not seem reasonable to pursue this aspect of the problem at this time.

The four zoeae and megalopa of *Pagurus longicarpus* were described in an earlier paper (Roberts, 1970). It was reported that these larvae did not correspond to those described by Thompson (1903) as *P. longicarpus*. It was suggested that Thompson had been working with *P. annulipes*. Nyblade (1970), based on his study of the larvae of the latter species, concluded that Thompson was indeed studying *P. annulipes*.

MATERIALS AND METHODS

Embryological development

Information on embryological development was obtained incidentally to the culture of eggs for various experiments. Eggs removed from the pleopods of a single female were incubated in filtered water at several salinities following the procedures developed by Costlow and Bookhout (1960). Quantitative observations, such as per cent hatch, were not attempted, but it was noted if development and hatching occurred and if there were any delay in hatching.

Larval development

Larvae for these experiments were hatched from females maintained in 5-liter battery jars containing water of 20‰ salinity. The water was replaced daily. Preliminary studies showed that eggs did not develop at all salinities included in this study. Further, no consistent difference was detected between mortality of larvae hatched at salinities from 15 to 30‰ *versus* larvae hatched at 20‰ and subsequently transferred to water with salinities from 15 to 30‰. Larvae were reared in water of 20‰ on a diet of nauplii of *Artemia* to the desired zoeal stage for each experiment. Tolerance tests were conducted in small finger bowls containing 200 ml water, 10 larvae per bowl. Fifty larvae were subjected to each test salinity in the range 10 to 30‰. All experiments were conducted at 20 ± 2° C, a temperature intermediate in the range tolerated by these larvae, though not necessarily optimal. This temperature is somewhat below the mean summer temperature in the York River estuary, but was convenient for the present study.

Four experiments of the above design were run, each experiment beginning with a different developmental stage. The experiment number corresponded with the zoeal stage used at the start of the test; thus Experiment 1 began with Zoea I, Experiment 2 with Zoea II and so forth. It was hoped in this manner to detect any increase (or decrease) in ability to tolerate reduced salinity as a function of zoeal stage and to detect any effect of prior culture conditions at the test salinity on each stage.

In the four experiments described above, tests were terminated after the molt to the megalopa since in most cases too few larvae reached this stage in healthy condition to produce meaningful results. An independent test, Experiment 5, was conducted with megalopae obtained from mass culture (100–200 larvae in 1000 ml, 20‰, 20° C). Since megalopae are aggressive and, in mass culture, frequently kill one another, compartmented boxes were used with one megalopa per compartment (50 ml). The same series of salinities was used as for tests with zoeae.

Table I

Hatching time as a function of incubation salinity and degree of development at start of incubation

Initial degree of development	Temperature (°C)	Days to hatching	
		20 to 30‰	15‰
Eyespot*	30	2	2
Eyestreak**	30	3	3
Eyestreak†	25	4	4
½ yolk***	25	5	6
"Early"****	25	7	9
"Early"	25	7	8

* Eyespot refers to eggs with dark brown or black circular eyespots and a beating heart.

** Eyestreak refers to eggs in which the eye is represented by a brown crescent-shaped spot.

*** ½ yolk refers to eggs with yolk occupying about ½ the egg capsule.

**** "Early" refers to eggs with at most a small white germinal disc apparent.

Further details concerning these stages are given in Coffin (1960).

† Artificial sea water (Rila mix) used in this experiment.

Water for all tests was prepared by diluting high salinity water collected at the branch laboratory at Wachapreague, Virigina. Water was collected from Finney Creek, filtered through a 1 μ filter, passed beneath ultraviolet lights, and collected in carboys. After transit to the main laboratory at Gloucester Point, the water was stored in large darkened carboys until used.

RESULTS

Embryological development

Development of embryos was followed at salinities of 10, 15, 20, 25 and 30‰. The results are summarized in Table I. Embryonic development was successfully completed at all salinities except 10‰ at which development proceeded for one or two days after which the yolk broke down. The eggs were greatly swollen and deteriorated rapidly. The same result was obtained even with late-stage eyed embryos placed in water of 10‰ salinity. Viable larvae hatched at 15‰ but generally 24–48 hours later than those hatched at higher salinities. The delay in development was more pronounced if eggs in an early stage of development were used. No effect was detected at salinities from 20 to 30‰.

Larval development: Survival

In all experiments, many deaths occurred just before, during, or immediately after a molt, especially the fourth molt. Larvae dying just prior to a molt showed anlagen of structures present in the succeeding stage beneath the old integument and separation of hypodermis from exuvium. These larvae apparently could not split the old integument, or could not remove themselves from it before they swelled. Larvae dying during the molt swelled while partly out of the exuvium. These larvae, with abdomen and part of the cephalothorax removed from the exuvium, were recorded as the stage following the molt. At the lowest salinities, the dorsum of the carapace was sometimes shed before the abdomen; these larvae were classed as the stage prior to the molt. Those dying immediately after the molt usually appeared weak at the last observation before death, and may have succumbed from the effort of completing the molt.

In Experiment 1, complete development to the megalopa was observed at all salinities from 15.5 to 30.5‰. No significant difference in survival was noted from 18.0 to 30.5‰ with 25 to 60% of the larvae reaching the megalopa. At 15.5‰, only 8% reached the megalopa. At 13.0‰ about 70% molted to Zoea II and 10% to Zoea III, but none to Zoea IV. At 10.5‰ only 2% survived to Zoea II and none to Zoea III (Fig. 1a). At salinities from 15.5 to 30.5‰, the slope of the survivorship curve was relatively constant up to Zoea IV but showed a marked increase for Zoea IV. At 13.0‰ there was a sharp increase in mortality for Zoea II.

Mortality observed immediately following transfer to 10.5 and 13.0‰ was due to the low salinity, not the salinity difference involved. Larvae transferred from 20.5 to 30.5‰, a salinity difference of equal magnitude, though in the opposite direction, did not exhibit significant mortality immediately after transfer. Further, 1 or 2 days after the larvae had been transferred to 10.5 and 13.0‰, mortality dropped to zero, and remained at zero until the next molt which was considerably delayed (see below).

Observations of larval development at salinities of 18.0 and 13.0‰ were omitted from the series in Experiment 2, but the results were essentially the same as in Experiment 1 with complete development to the megalopa observed from 15.5 to 30.5‰. From 20.5 to 30.5‰, 35 to 55% survived to the megalopa; at 15.5‰, only 4%. No larvae reached Zoea III at 10.5‰. Most larvae in this latter salinity died shortly after transfer, the remainder just prior to the molt. The slopes of the survivorship curves are essentially constant up to Zoea IV at all salinities except 10.5‰ (Fig. 1b).

Complete development occurred over the salinity range 13.0 to 30.5‰ in Experiment 3. From 18.0 to 30.5‰, 60 to 83% reached the megalopa, at 15.5‰, 40% and at 13.0‰, 5% (Fig. 1c). Thus at 15.5‰ there was an apparent increase in survival to the megalopa from 4–8% to 40% while at 13.0‰, the increase is from 0% to 5%.

Megalopae were obtained in every salinity including 10.5‰ in Experiment 4. Better than 80% survived at all salinities from 13.0 to 30.5‰, and 5.3% at 10.5‰ (Fig. 1d).

While it might seem that there is a marked increase in salinity tolerance with increasing developmental age, a comparison of the family of survivorship curves

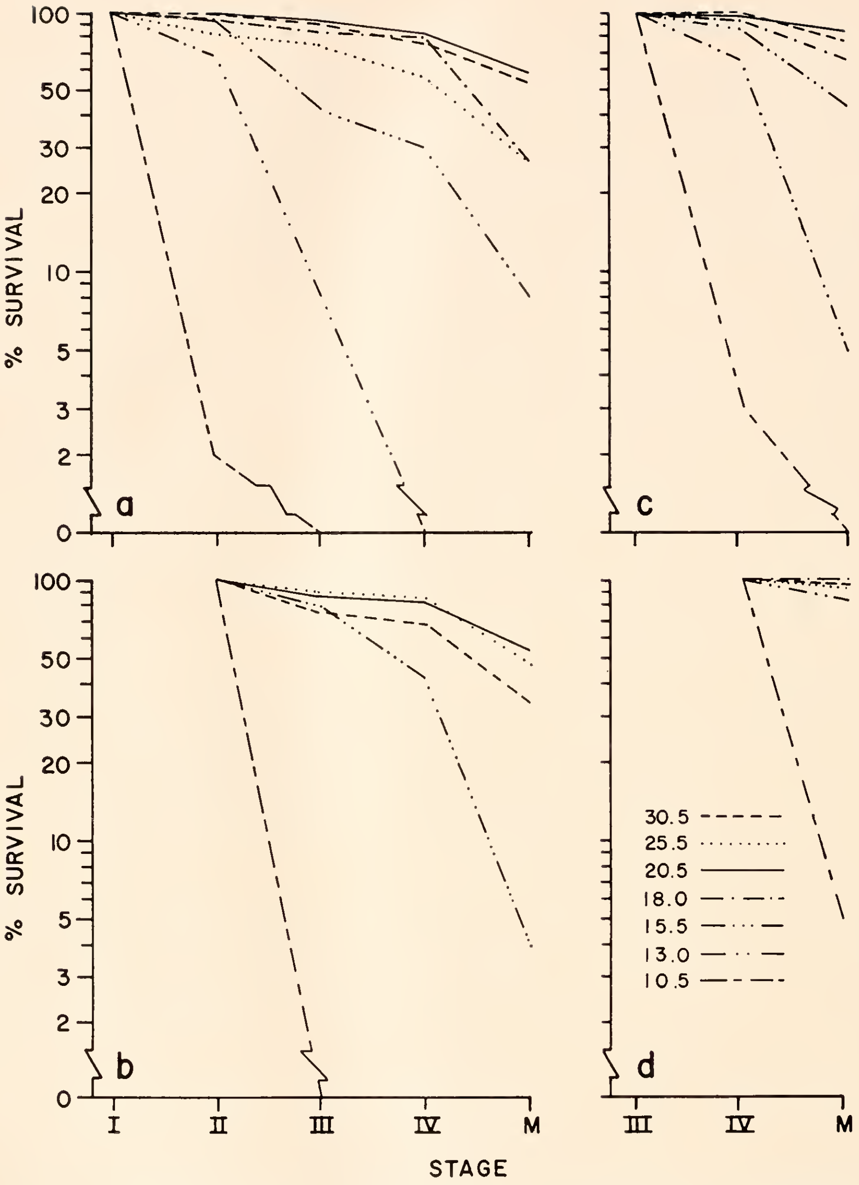

Figure 1. Survivorship curves for the salinity tolerance experiments; (a) Experiment 1, (b) Experiment 2, (c) Experiment 3, (d) Experiment 4. In Experiment 4, the results were identical for 30.5 and 20.5 ‰ and for 18.0 and 15.5 ‰.

for the four experiments suggests that this is not the case. The curves for each experiment are exactly like those of Experiment 1 if the latter are displaced an appropriate number of developmental stages to the right.

In order to compare the mortality in each zoeal instar in the various experiments, the per cent mortality was calculated as follows:

$$\% M = \frac{n_i - n_{i+1}}{n_i} \times 100$$

where

n_i = number of Zoea i
n_{i+1} = number of Zoea $i + 1$

These values are arrayed in Table II. Assuming that the per cent mortalities so obtained are binomially distributed, it is possible to estimate 95% confidence limits from tables for the appropriate sample size and point estimate (Diem, 1962). From a consideration of the 95% confidence limits and the point estimates for each cell in the array in Table II, I was led to the conclusion that three values were not adequate estimates of the mortality resulting from salinity. These values are enclosed in brackets.

The initial zoeal stage used in each experiment had a lower per cent mortality than succeeding stages. Indeed, no difference could be detected in the per cent mortality of the initial stage of each experiment at any given salinity. This can be seen by comparing the per cent mortality for the initial stage used in each experiment at any given salinity in Table II. This results from the selection of only healthy larvae, *i.e.,* those showing a strong positive light response, in setting up each experiment. The increase in mortality in succeeding stages, which usually was not very great except in Zoea IV, reflects at least in part the fact that some larvae remaining after the initial molt were not healthy.

There was no significant difference in per cent mortality for any given zoeal stage over the salinity range 18.0 to 30.5‰. This can be seen by comparing values for any given stage, *e.g.,* Zoea III, over the entire range of salinity. Per cent mortality for the initial stage in each experiment at 15.5‰ was in the same range as that of 18.0 to 30.5‰, but many larvae were weakened as indicated by the significant increase in mortality in the succeeding stage. The same effect was noted at 13.0‰ but at a significantly higher mortality level. If tolerance of reduced salinity had improved with increasing developmental age, one would expect a marked improvement in survival of later stages in 15.5‰ in the later experiments.

Per cent mortality for the megalopa (Experiment 5) was less than 6% from 18.0 to 30.5‰, which agrees with the results obtained for the zoeal stages. At lower salinities per cent mortality increased rapidly to 100% at 10.5‰. At 15.5‰ the per cent mortality was significantly higher than at higher salinities with most deaths occurring near the time of molt to the juvenile. Deaths at 13.0‰ also were associated with molting, while those at 10.5‰ were associated with the stress produced by transfer to this salinity. This may represent a slightly lessened ability of the megalopa to tolerate reduced salinity.

Larval development: Intermolt duration

The mean time after hatching to each zoeal stage, in days, was calculated for all experiments. The number molting during each 24-hour period was asso-

TABLE II

Per cent mortality for the zoeal stages and megalopae at each salinity

Salinity	Experiment number	Zoeal stages				
		I	II	III	IV	Megalopa
30.5	1	2.0	8.2	15.5	31.4	/
	2	—	[21.6]	12.8	50.0	/
	3	—	—	0.0	22.5	/
	4	—	—	—	2.6	/
	5	—	—	—	—	5.6
25.5	1	[18.0]	9.8	[24.3]	51.8	/
	2	—	10.0	6.7	57.2	/
	3	—	—	—	—	/
	4	—	—	—	5.3	/
	5	—	—	—	—	0.0
20.5	1	2.0	7.1	11.0	29.0	/
	2	—	14.0	4.7	34.9	/
	3	—	—	2.5	15.4	/
	4	—	—	—	2.6	/
	5	—	—	—	—	2.8
18.0	1	6.0	10.6	4.8	67.5	/
	2	—	—	—	—	/
	3	—	—	5.0	31.6	/
	4	—	—	—	0.0	/
	5	—	—	—	—	5.6
15.5	1	7.0	54.8	28.6	73.5	/
	2	—	20.0	47.6	90.5	/
	3	—	—	15.0	50.0	/
	4	—	—	—	0.0	/
	5	—	—	—	—	22.2
13.0	1	32.0	88.3	100.0	/	/
	2	—	—	—	—	/
	3	—	—	35.0	92.0	/
	4	—	—	—	15.8	/
	5	—	—	—	—	66.7
10.5	1	98.0	100.0	/	/	/
	2	—	100.0	/	/	/
	3	—	—	97.6	100.0	/
	4	—	—	—	94.7	/
	5	—	—	—	—	100.0

ciated with the mean time after hatching for that period for the purposes of calculation. This procedure assumes that the frequency of molting was normally distributed through time.

Mean post-hatching time, range of post-hatching time, and number of larvae involved for each zoeal stage are presented in Table III and Figure 2. Intermolt duration for each zoeal stage was then calculated as the difference between the mean post-hatching times from Experiment 1, except in cases where no information was available. When information was available from the other experiments, it was used instead.

Rigorous statistical comparison of mean post-hatching time for each molt was not possible because the observation intervals were very large relative to the interval between the first and last zoea completing a given molt. Such intensive grouping causes a gross underestimate of the true variance, thereby increasing the chance of rejecting the null hypothesis that the mean post-hatching times were equal, when it should be accepted. Intensive grouping also tends to bias the estimate of the mean in an indeterminate manner since there is no way of knowing in any given case whether the larvae molted at the beginning or end of an observation period.

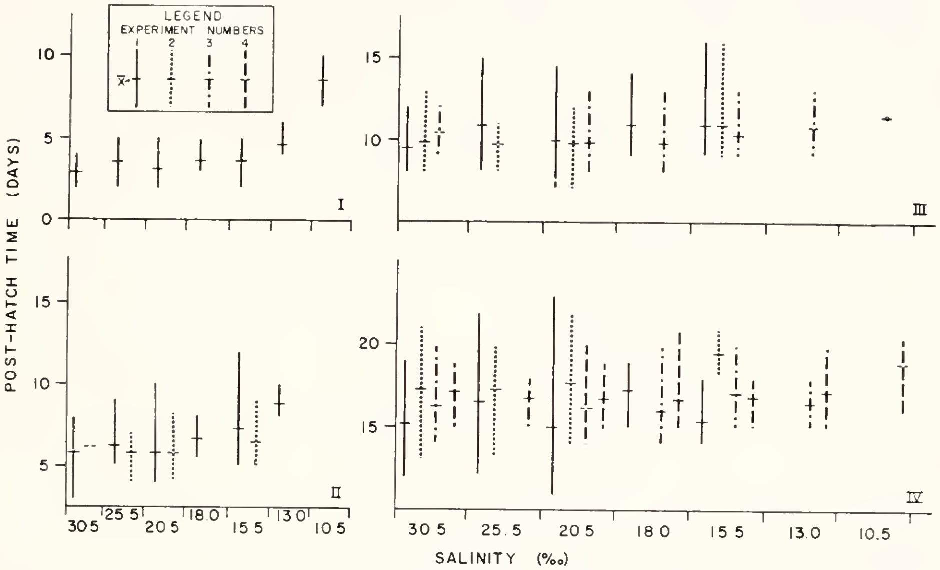

FIGURE 2. Range and mean post-hatch time for each zoeal stage.

The intermolt duration over the salinity range 15.5 to 30.5‰ was 3.0 to 3.5 days for Zoea I, 2.7 to 3.8 days for Zoea II, 3.5 to 4.6 days for Zoea III, 4.6 to 6.4 days for Zoea IV. Intermolt duration increased slightly at 13.0 and 10.5‰.

Mean post-hatching times derived from Experiments 1 to 4 show very close agreement at each salinity except in some cases for the molt from Zoea IV to megalopa (Fig. 2). The poor agreement in this case results in part from the small numbers of larvae involved.

Intermolt duration for the megalopa was obtained directly since the protocol for this experiment was slightly different. The intermolt duration was 6.5 to 7.5 days at salinities from 18.0 to 30.5‰, increasing to 8 and 9 days at 15.5 and 13.0‰, respectively. The increased intermolt duration for Zoea IV and the

TABLE III

Mean post-hatching times to the end of each zoeal stage and intermolt durations for each larval stage

Developmental stage	Experiment number		Salinity, ‰						
			30.5	25.5	20.5	18.0	15.5	13.0	10.5
Zoea I	1	x̄	2.95	3.50	3.04	3.52	3.54	4.59	8.50
		r	(2–4)	(2–5)	(2–5)	(3–5)	(2–5)	(4–6)	(7–10)
		n	49	41	98	47	93	34	2
	Intermolt duration		3.0	3.5	3.1	3.5	3.5	4.6	8.5
Zoea II	1	x̄	5.88	6.23	5.82	6.60	7.31	8.75	—
		r	(3–8)	(5–9)	(4–10)	(6–8)	(5–12)	(8–10)	—
		n	45	37	91	42	42	4	—
	2	x̄	6.04	5.81	5.76	—	6.40	—	—
		r	(4–8)	(4–7)	(4–8)	—	(5–9)	—	—
		n	39	45	43	—	40	—	—
	Intermolt duration		2.9	2.7	2.7	3.1	3.8	4.2	—
Zoea III	1	x̄	9.50	10.81	9.94	10.95	10.80	—	—
		r	(8–12)	(8–15)	(7–14)	(9–14)	(9–16)	—	—
		n	38	28	81	40	30	—	—
	2	x̄	9.85	9.67	9.70	—	10.83	—	—
		r	(8–13)	(8–11)	(8–12)	—	(9–16)	—	—
		n	34	42	41	—	21	—	—
	3	x̄	10.40	—	9.81	9.71	10.24	10.81	11.50
		r	(9–12)	—	(8–13)	(8–13)	(9–13)	(9–13)	(12)
		n	40	—	39	38	34	26	1
	Intermolt duration		3.6	4.6	4.1	4.4	3.5	(5.0)	(5.7)
Zoea IV	1	x̄	15.12	16.58	15.01	17.35	15.38	—	—
		r	(12–19)	(12–22)	(11–23)	(15–19)	(14–18)	—	—
		n	26	13	58	13	8		—
	2	x̄	17.26	17.19	17.69	—	19.50	—	—
		r	(13–21)	(13–20)	(14–22)	—	(18–21)	—	—
		n	17	24	27	—	2	—	—
	3	x̄	16.40	—	16.17	16.04	17.09	16.50	—
		r	(14–20)	—	(14–20)	(14–20)	(15–20)	(15–18)	—
		n	31	—	33	26	17	2	—
	4	x̄	17.12	16.78	16.82	16.74	16.84	17.09	19.00
		r	(15–19)	(15–18)	(15–19)	(15–21)	(15–18)	(15–20)	(16–21)
		n	37	36	37	38	38	32	2
	Intermolt duration		5.6	5.8	5.1	6.4	4.6	(5.7)	(8.1)
Megalopa*	5	x̄	7.35	6.47	6.55	7.04	8.18	9.08	—
		r	(6–8)	(5–8)	(5–9)	(5–9)	(6–10)	(7–11)	—
		n	34	36	35	34	28	12	—

* Values for this stage are given as intermolt duration, in days, rather than post-hatching time, in days, as for all other stages.

megalopa compared to earlier zoeal stages has been noted before in *Petrochirus diogenes* (Provenzano, 1968).

DISCUSSION

The notion that certain life history stages are more subject to limitation by abiotic environmental factors such as salinity, was first enunciated by Shelford (1915). Since then, considerable evidence has been amassed demonstrating that younger stages tend to be less tolerant than adults. Among decapod crustaceans, perhaps the best documented example is the blue crab, *Callinectes sapidus,* which can tolerate salinities from fresh to oceanic as an adult, but must return to water with a salinity in excess of 15‰ for hatching of the eggs. Complete larval development occurs only at 20‰ and above (Sandoz and Rogers, 1944; Costlow and Bookhout, 1959; Costlow, 1967). Other examples from various phyla may be found in recent reviews by Kinne (1964, 1966) and other papers in the literature.

Salinity tolerance of decapod embryos has received only cursory attention. Broekhuysen (1936) cultured *Carcinus maenas* eggs at salinities from 10 to 50‰. At salinities from 20 to 40‰, 16° C, and 25 to 40‰, 10° C, complete embryonic development occurred, while at salinities above and below this range, development occurred to a degree, but hatching was not observed. At 10‰, no embryonic development was detected. Tolerance of each larval stage is unknown, but the adult tolerance is from 4 to 34‰ salinity (or above). Hatching of *Hepatus epheliticus* has been observed at all salinities from 20 to 40‰ which is a greater range than tolerated by either larvae or adults (Costlow and Bookhout, 1962). In the present study, hatching of *P. longicarpus* was observed from 15 to 30‰ at 25 and 30° C. This is very close to the larval tolerance but probably slightly less than the adult tolerance, although the latter is not precisely known.

It is concluded that each species has a unique range of salinities suitable for embryonic development and hatching which bears no *a priori* relationship to the tolerance of adults or larvae. The egg is by no means the most sensitive life history stage in all decapod crustaceans.

Salinity tolerances for the larvae of several brachyuran species have now been studied in detail, in several cases in combination with temperature tolerances. *Rhithropanopeus harrisii* can develop over the salinity range 1 to 40‰ with optimal development between 15 and 30‰ at 20 to 30° C (Chamberlain, 1962; Costlow *et al.,* 1966). However, field observations from the Miramichi Estuary indicate that *Rhithropanopeus* larvae are usually restricted to water with a salinity less than 23.5‰ (Bousfield, 1955). This probably reflects the distribution of the adults which is similarly restricted. *Sesarma cinereum* has a different optimal salinity range for each larval stage, with Zoea IV most sensitive (optimum: 15–25‰, 30° C, 22–28‰, 20° C). The total range tolerated is much greater than the optimal range (Costlow *et al.,* 1960). *Panopeus herbstii* larvae tolerate a broader salinity range than *S. cinereum,* but not as broad a range as *R. harrisii.* In *Panopeus,* no stage is particularly sensitive to salinity (Costlow *et al.,* 1962). *Callinectes,* an estuarine species like those above, was mentioned previously in this regard. *Hepatus epheliticus,* a polystenohaline crab, develops completely over only a narrow salinity range of 30 to 35‰ (Costlow and Bookhout, 1962). The terrestrial brachyuran, *Cardisoma guanhumi,* exhibits complete development from 15 to 45‰. The special adaptive osmoregulatory mechanisms of the adult are not developed in the larvae, which have a tolerance range comparable to an estuarine species (Costlow and Bookhout, 1968).

Lance (1964) used a different procedure for studying tolerance levels of *Carcinus maenas* Zoea I and megalopa and *Pisidia longicornis* Zoea I and II and megalopa. She transferred larvae to containers with water of varying salinities and counted the number dead after 20 hours, at which time the experiment was terminated. *Carcinus maenas* Zoea I was less tolerant than the megalopa, with 50% mortality at salinities below 12.6 and 9.9‰ respectively. The two zoeal stages of *Pisidia longicornis* were identical in tolerance capacity with more than 50% mortality at salinities below 14.4‰. The megalopa is less tolerant, with more than 50% mortality at salinities below 20.7‰.

Lance (1964) measured the acute effects of salinity during the intermolt period rather than the chronic effects of prolonged exposure to various salinities. It has been observed by many investigators that most deaths attributable to salinity occur about the time of ecdysis unless the salinity is extreme relative to the tolerance ability of the given species. At ecdysis, major changes in the cuticle in effect lower the defenses of the animal. Tolerance ranges based on studies of acute salinity effects tend to be broader than the actual tolerance range. To understand the effect of salinity on distribution, one must know the true tolerance range based on studies of chronic effects of salinity.

Of the pagurid species studied previously, *Pagurus samuelis* was observed to develop to Zoea IV only from 22.5 to 35‰, and to the megalopa only at 29‰. No juveniles were obtained in salinity tolerance experiments because the temperature exceeded the lethal limit late in the experiment (Coffin, 1958). *Pagurus bernhardus* developed completely to the juvenile only at 30 and 35‰. A small percentage of megalopae were obtained at 25‰ but this result was not duplicated in another experiment (Bookhout, 1964).

Pagurus longicarpus developed over a much wider salinity range than either *Pagurus* species mentioned above. Of the three species, this is the only one penetrating estuaries to a significant extent. The optimal salinity range is broad, at least from 18 to 30.5‰ and probably above. Over this range there was no detectable difference in mortality or intermolt duration. Tolerance of reduced salinity is the same for all four zoeal stages, slightly less for the megalopa. The optimal salinity range is only slightly less than the total salinity range permitting complete development and is one of the broadest optimal salinity ranges known.

Coffin (1958) indicated the possibility of cannibalism during zoeal stages in his experiments. Bookhout (1964) also concluded there was cannibalism, as much as 15 to 35% at 35‰, decreasing to 2.5 to 27.5% at 25‰ in his experiments. In the present study, no evidence of cannibalism has been observed during the zoeal stages even in large mass cultures with larval densities of 1 larva per 5 ml. Maximum density in the tolerance experiments was only 1 larva per 20 ml.

Cannibalism has been observed among megalopae in mass culture, necessitating rearing them in isolation. Thompson (1903) and Bookhout (1964) reported high mortality rates for megalopae not provided with shells and low mortality rates for those with shells. Since both authors reared larvae in mass cultures, megalopae with shells were less likely to be cannibalized than those without. In addition, Bookhout (1964) was dealing with sample sizes too small to draw very firm conclusions. In an independent set of experiments to be described in a subsequent paper, no difference was observed in mortality rate of megalopae provided with shells and those without when the tests were conducted with each megalopa isolated in compartmented boxes. The results of the experiment on salinity tolerance of megalopae are therefore not subject to criticism because no shells were provided for the larvae.

It is not possible to compare in detail larval and adult tolerances for *P. longicarpus,* as the latter have not been studied. However, it is known that for the adults, the haemolymph has the same osmotic concentration as the medium over the salinity range 18 to 43‰ (Kinne, Shirley and Meen, 1963).

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LITERATURE CITED

Banse, K., 1956. Über den Transport von meroplanktischen Larven aus dem Kattegat in die Kieler Bucht. *Ber. Deut. Wiss. Komm. Meeresforsch.,* **14**: 147–164.

Banse, K., 1959. Die Vertikalverteilung planktischer Copepoden in der Kieler Bucht. *Ber. Deut. Wiss. Komm. Meeresforsch.,* **15**: 357–388.

Bary, B. M., 1963a. Temperature, salinity and plankton in the eastern North Atlantic and coastal waters of Britain, 1957. I. The characterization and distribution of surface waters. *J. Fish. Res. Board, Can.,* **20**: 789–826.

Bary, B. M., 1963b. Temperature, salinity and plankton in the eastern North Atlantic and coastal waters of Britain, 1957. II. The relationships between species and water bodies. *J. Fish. Res. Board, Can.,* **20**: 1031–1065.

Bary, B. M., 1963c. Temperature, salinity and plankton in the eastern North Atlantic and coastal waters of Britain, 1957. III. The distribution of zooplankton in relation to water bodies. *J. Fish. Res. Board, Can.,* **20**: 1519–1548.

Bookhout, C. G., 1964. Salinity effects on the larval development of *Pagurus bernhardus* (L.) reared in the laboratory. *Ophelia,* **1**: 275–294.

Bousfield, E. L., 1955. Ecological control of the occurrence of barnacles in the Miramichi Estuary. *Bull. Nat. Mus. Canada,* **137**: 1–69.

Boyd, C. M., and M. W. Johnson, 1963. Variations in the larval stages of a decapod crustacean, *Pleuroncodes planipes* Stimpson (Galatheidae). *Biol. Bull.,* **124**: 141–152.

Broekhuysen, G. J., Jr., 1936. On development, growth, and distribution of *Carcinides maenas* (L.). *Arch. Neerl. Zool.,* **2**: 257–399.

Coffin, H. G., 1958. The laboratory culture of *Pagurus samuelis* Stimpson. *Walla Walla Coll. Publ.,* **22**: 1–5.

COFFIN, H. G., 1960. The ovulation, embryology, and developmental stages of the hermit crab, *Pagurus samuelis* Stimpson. *Walla Walla Coll. Publ.*, **25**: 1–30.

CHAMBERLAIN, N. A., 1961. Studies on the larval development of *Neopanope texana sayi* (Smith) and other crabs of the Zanthidae (Brachyura). *Chesapeake Bay Institute, Johns Hopkins Univ., Tech Rep.*, **22**: 1–35.

CHAMBERLAIN, N. A., 1962. Ecological studies of the larval development of *Rhithropanopeus harrisii* (Xanthidae, Brachyura). *Chesapeake Bay Institute, Johns Hopkins Univ., Tech. Rep.*, **28**: 1–47.

COSTLOW, J. D., JR., 1967. The effect of salinity and temperature on survival and metamorphosis of megalops of the blue crab, *Callinectes sapidus* Rathbun. *Helgolaender Wiss. Meeresunters.*, **15**: 84–97.

COSTLOW, J. D., JR., AND C. G. BOOKHOUT, 1959. The larval development of *Callinectes sapidus* Rathbun reared in the laboratory. *Biol. Bull.*, **116**: 373–396.

COSTLOW, J. D., JR., AND C. G. BOOKHOUT, 1960. A method for developing Brachyuran eggs *in vitro*. *Limnol. Oceanogr.*, **5**: 212–215.

COSTLOW, J. D., JR., AND C. G. BOOKHOUT, 1962. The larval development of *Hepatus epheliticus* (L.) under laboratory conditions. *J. Elisha Mitchell Sci. Soc.*, **78**: 113–125.

COSTLOW, J. D., JR., AND C. G. BOOKHOUT, 1968. The effect of environmental factors on the development of the land-crab *Cardisoma guanhumi* Latreille. *Amer. Zool.*, **8**: 397–410.

COSTLOW, J. D., JR., C. G. BOOKHOUT AND R. MONROE, 1960. The effect of salinity and temperature on larval development of *Sesarma cinereum* (Bosc) reared in the laboratory. *Biol. Bull.*, **118**: 183–202.

COSTLOW, J. D., JR., C. G. BOOKHOUT AND R. MONROE, 1962. Salinity-temperature effects on the larval development of the crab, *Panopeus herbstii* Milne-Edwards, reared in the laboratory. *Physiol. Zool.*, **35**: 79–93.

COSTLOW, J. D., JR., C. G. BOOKHOUT AND R. MONROE, 1966. Studies on the larval development of the crab, *Rhithropanopeus harrisii* (Gould). I. The effect of salinity and temperature on larval development. *Physiol. Zool.*, **39**: 81–100.

DIEM, K. (Ed.), 1962. *Documenta Geigy, Scientific Tables.* [6th Edition] Geigy Pharmaceuticals, Ardsley, New York, 778 pp.

KINNE, O., 1964. The effects of temperature and salinity on marine and brackish water animals. II. Salinity. *Oceanogr. Mar. Biol., Ann. Rev.*, **2**: 281–339.

KINNE, O., 1966. Physiological aspects of animal life in estuaries with special reference to salinity. *Netherlands J. Sea Res.*, **3**: 222–244.

KINNE, O., E. K. SHIRLEY AND H. E. MEEN, 1963. Osmotic responses of hermit crabs (*Pagurus longicarpus* Say) exposed to various constant temperatures and salinities. *Crustaceana*, **5**: 317.

LANCE, J., 1964. The salinity tolerances of some estuarine planktonic crustaceans. *Biol. Bull.*, **127**: 108–118.

NYBLADE, C., 1970. Larval development of *Pagurus annulipes* (Stimpson, 1862) and *Pagurus pollicaris* Say, 1817 reared in the laboratory. *Biol. Bull.*, **139**: 557–573.

PROVENZANO, A. J., JR., 1968. The complete larval development of the West Indian hermit crab *Petrochirus diogenes* (L.) (Decapoda, Diogenidae) reared in the laboratory. *Bull. Mar. Sci.*, **18**: 143–181.

ROBERTS, M. H., JR., 1970. Larval development of *Pagurus longicarpus* Say reared in the laboratory, I. Description of larval instars. *Biol. Bull.*, **139**: 188–202.

SANDOZ, M., AND R. ROGERS, 1944. The effect of environmental factors on hatching, moulting, and survival of the zoea larvae of the blue crab, *Callinectes sapidus* Rathbun. *Ecology*, **25**: 216–228.

SHELFORD, V. E., 1915. Principles and problems of ecology as illustrated by animals. *J. Ecol.*, **3**: 1–23.

THOMPSON, M. T., 1903. The metamorphosis of the hermit crab. *Proc. Boston Soc. Natur. Hist.*, **31**: 147–209.

THORSON, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates. *Medd. Dan. Fisk. Havunders*, **4**: 1–523.

Reference: *Biol. Bull.*, **140**: 117–124. (February, 1971)

BIPOLAR HEAD REGENERATION IN PLANARIA INDUCED BY CHICK EMBRYO EXTRACTS [1]

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When a planarian is transected the anterior cut end will regenerate a head while the posterior end will give rise to a tail. Child (1941), postulated in his axial gradient theory that quantitive axial gradients of physiological activity somehow account for axial patterns of differentiation. This theory predicts that the higher level of physiological activity at the anterior cut end leads to the formation of the head at that site. An alternative suggestion is that polarity is maintained by the presence of specific inhibitors which similarly follow an axial gradient pattern. When a planarian is decapitated, the brain forms before the eye spots or auricles. However, regeneration of the brain does not occur if another brain is present (Morgan, 1902; Rand and Ellis, 1926; and Miller, 1938). Such effects could be due to specific inhibitors or they could result from a metabolic competition. Decapitated planarians reared in water containing centrifugal supernatants of head homogenates had smaller brains, or the brains were absent, suggesting there are specific inhibitor substances (Lender, 1960).

The aim of this investigation was to determine if polarity is determined by the presence of specific inhibitors or if it is the function of an axial gradient of physiological activity. Regeneration was allowed to occur in the presence of centrifugal supernatants of either heads or tails of planaria, *Dugesia dorotocephala*, to determine if these fractions possess inhibitory activity. In order to test the idea that competitive metabolic processes may play a role in determining polarity, small cut pieces of planaria were cultured in chick embryo extract and ultrafiltrate to see if these nutrients provided in these preparations could alter the normal regeneration polarity by inducing the formation of bipolar heads.

MATERIALS AND METHODS

Planarians, *Dugesia dorotocephala,* which were 1–2 centimeters in length were maintained in aerated tap water in covered containers containing 100 units of penicillin and streptomycin per milliliter and were starved for at least 5–7 days prior to use in an experiment. Before cutting, the worms were washed several times with boiled tap water, then while beneath a shallow layer of water they were exposed to bacteriocidal ultraviolet light for five minutes and then cut with sterile single edge razor blades. In obtaining the centrifugal supernatants for the inhibitor experiments the worms were cut into three equal parts, thus providing the head, mid-body and tail fractions. The middle piece always included the

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pharynx. The protein concentrations of the centrifugal supernatants were determined by the Lowry method (Lowry, Rosebrough, Farr and Randall, 1951). The chick embryo extracts used in these experiments were obtained in the frozen state from Grand Island Biological Company. The effect of the chick embryo fractions upon DNA synthesis in pieces of planarians was tested by exposing pooled segments of planaria to 25 μ c/ml of H^3-thymidine in 2 ml of the chick embryo extract solutions and in a 1/10 dilution of a balanced salt medium (Niu-Twitty, 1953). At the conclusion of the incubation period the worms were washed five times with boiled tap water. The worms were then homogenized in cold 7% trichloroacetic acid (TCA) and the pellet washed three times by centrifugation. The residues were extracted twice with 1:1 ethanol-ether, twice with 1:4 ethanol-ether and once with ether alone to remove lipids; the residues were then dried. The diphenylamine method (Dische, 1955) was used to determine DNA concentration and levels of labeled DNA were estimated with a liquid scintillation counter.

RESULTS

Experiments with centrifugal supernatants

The head, tail and midbody centrifugal supernates used for the inhibitor experiments were prepared by homogenizing at 4° C in aerated tap water containing 50 units of penicillin and sterptomycin per milliliter. Homogenates were centrifuged at 10,000 × *g* for 30 minutes at 4° C and the supernatants were collected. The supernatants were passed through sterile millipore filters of 0.45 and 0.22 μ pore diameter before use. Heads were removed from a group of 25 worms by cutting just posterior to the auricles and the decapitated worms were exposed for 9 days at 20° C to supernatants of homogenized head, tail and midbody sections at concentrations between 2 and 100 pieces per two milliliters of homogenation medium. In terms of protein concentration, cut planaria were exposed to centrifugal supernatants from head regions with a protein concentration of 6.05 μg/ml to 605 μg/ml and to centrifugal supernatants from tail regions with protein concentrations of 4.58 μg/ml to 458 μg/ml. Fresh supernatants were prepared daily and the media changed each day of the nine day exposure period. Control and test worms were observed daily for the appearance of eyespots and auricles. After nine days of culture, test and control worms were fixed at which time both eye spots and auricles had appeared. They were then embedded in paraffin, 5 μ serial sections made and sections were stained with 0.1% cresyl violet.

All planaria exposed to head and tail concentrations of 2 through 87 pieces per two milliliters survived the nine days of incubation and regenerated normal eyespots and auricles. Tail and head concentrations of 100 pieces/2 ml killed test worms within 48 hours after first exposure. In control and test worms incubated in non-lethal supernatant concentrations, auricles appeared between 48–72 hours, detectable eyespots between 60–96 hours, fully discernable eyespots between 85–120 hours and fully regenerated heads by 6 days. There was no significant delay in time of appearance of the eyespots in the centrifugal supernatant fractions. Serial sections of control and test worms showed the presence of brains of equal length (175 ± 5 μ) in both the control and test worms (Fig. 1).

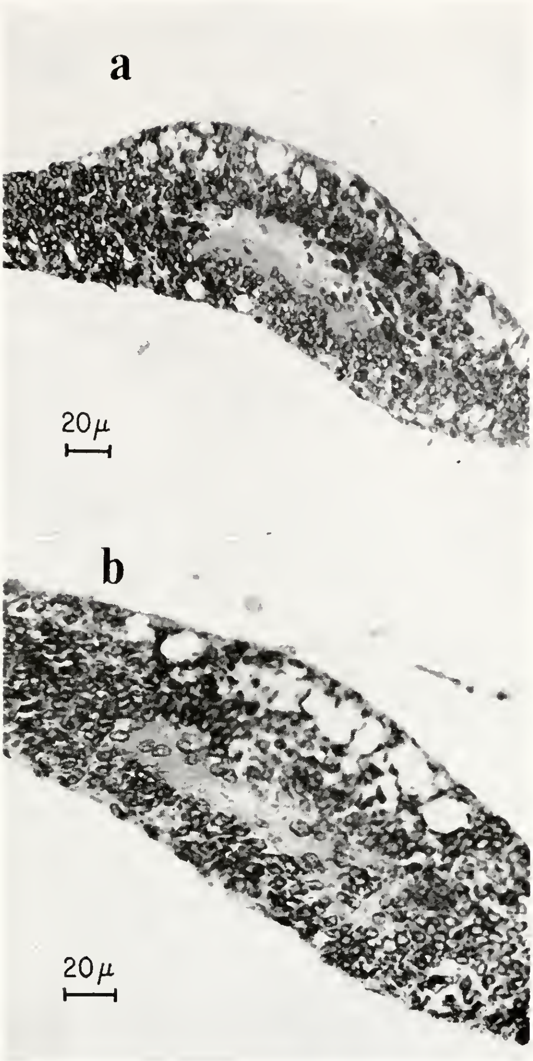

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FIGURE 1. Transverse sections of heads that formed after 9 days of regeneration in (a) bubbled tap water and (b) a centrifugal supernate obtained by homogenizing 50 planarian heads in 2 ml of bubbled tap water. The more lightly stained tissues in the center of the sections are the brains.

Effect of chick embryo extract on polarity

In a preliminary experiment, to determine what body levels and which nutrient medium produced bipolars, the planaria were transversely cut at 8 body levels (Fig. 2) and the cut segments of worms allowed to regenerate in chick embryo extract and chick embryo extract ultrafiltrate. Regenerating pieces from posterior levels 5 and 6 cultured in 1% chick embryo extract and 1% chick embryo extract

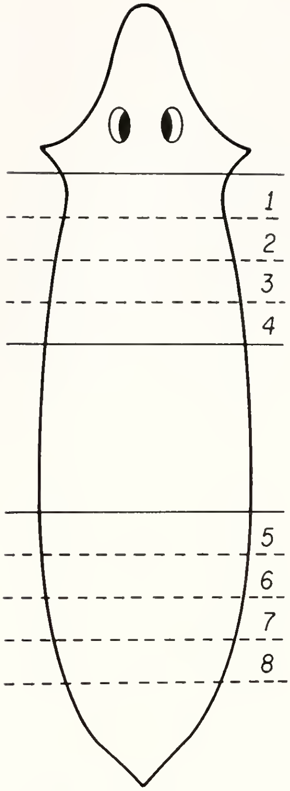

FIGURE 2. The levels at which worms were cut for the experiments with chick embryo extract.

ultrafiltrate in 1/10 Niu-Twitty saline (Niu and Twitty, 1953) were the only levels showing bipolar head regeneration, *i.e.*, heads forming at each end of the cut segments of the worms. Segments of worms from regions 1 to 4 and 7 to 8 regenerated heads at the anterior end and tails at the posterior ends. No bipolar heads arose in pieces cultured in saline alone and these pieces formed heads at the anterior end surface and tails at the posterior end.

In further experiments, twenty sections each of posterior body levels 5 and 6 from worms starved for 5, 10, 20 and 30 days were cultured in saline and nutrient media. The sections were exposed to test solutions for 4 days, after which regeneration was completed in 1/10 saline (Table I). No bipolar head regeneration occurred in the short posterior pieces from worms starved 5–10 days.

However, 6 bipolar heads out of 26 cut pieces regenerated from cut posterior level 5 pieces and 8 bipolar heads out of 21 cut pieces were formed in posterior level 6 cut pieces in nutrient media from worms starved for 20 days. After 30 days of starvation, 9 of 34 level 5 pieces formed bipolar heads in nutrient media, while 7 of 25 level 6 pieces in nutrient media regenerated bipolar heads. None of the posterior level pieces produced bipolar heads when the medium was 1/10 Niu Twitty saline (Table I). A typical regenerate with a bipolar head is shown in Figure 3.

TABLE I

Effect of nutrient media on incidence of bipolar head formation

Medium	Level of worm	$\frac{\text{Number of bipolars}}{\text{Number of surviving sections}} = \%$			
		Days of starvation			
		5	10	20	30
(1.) 1/10 Niu-Twitty saline +1% chick embryo extract	5	0/20 = 0	0/19 = 0	4/10 = 40%	4/16 = 25%
(2.) 1/10 Niu-Twitty saline +1% chick embryo extract ultrafiltrate	5	0/19 = 0	0/19 = 0	2/16 = 12.5%	5/18 = 27.8%
(3.) 1/10 Niu-Twitty saline	5	0/20 = 0	0/18 = 0	0/16 = 0	0/14 = 0
(1.) 1/10 Niu-Twitty saline +1% chick embryo extract	6	0/20 = 0	0/19 = 0	3/5 = 60%	3/12 = 25%
(2.) 1/10 Niu-Twitty saline +1% chick embryo extract ultrafiltrate	6	0/20 = 0	0/19 = 0	5/16 = 31.25%	4/13 = 30.7%
(3.) 1/10 Niu-Twitty saline	6	0/20 = 0	0/17 = 0	0/19 = 0	0/15 = 0

Isotopic experiments with worms exposed to chick embryo extract

It is possible that the chick embryo extract causes bipolar head regeneration in short posterior pieces from starved planaria by stimulating cell division. In order to determine if the chick embryo extracts had stimulated DNA synthesis and cell division in the regenerating sections an experiment using H^3-thymidine was performed. One hundred and fifty worms starved for 30 days were cut into

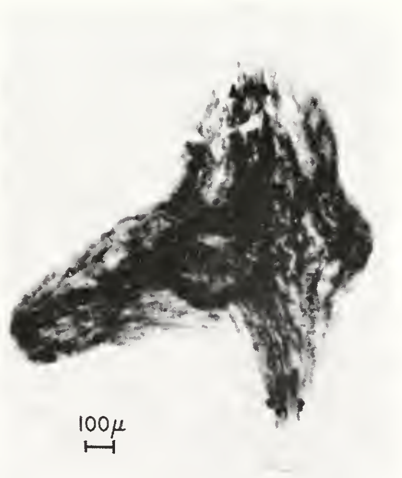

FIGURE 3. A posterior section of a worm which regenerated a head at each end after culture in 1% chick embryo extract.

head (levels 1–4), midbody (between levels 4 and 5) and tail pieces (levels 5–8) (Fig. 2). Fifty such sections of each level were each incubated in two nutrient media and saline. To each of the media H^3-thymidine was added to a final concentration of 25 μ c/ml and cut worms were incubated for 24 hours. The results of this experiment clearly demonstrate a stimulation of DNA synthesis of all three levels of the pooled cut worms which had been cultured in nutrient medium (Table II). There was a greater stimulation of DNA synthesis at middle and posterior levels than at anterior levels. Those cut pieces giving rise to bipolar heads were found using posterior levels of cut worms (Table I).

TABLE II

Effect of nutrient media on DNA syntheses in planaria starved for 30 days. Fifty cut pieces were incubated in 25 μ c/ml H^3-thymidine for 46 hours

$$\frac{\text{CPM}}{\text{MG DNA}}$$

Medium	Level of worm			% stimulation DNA synthesis compared to controls		
	Anterior	Middle	Posterior	Anterior	Middle	Posterior
(1.) 1/10 Niu-Twitty saline + 1% chick embryo extract	21,400	16,756	14,872	19.4	37.9	32.1
(2.) 1/10 Niu-Twitty saline + 1% chick embryo extract ultrafiltrate	18,460	15,539	12,600	13.7	33.1	19.8
(3.) 1/10 Niu-Twitty saline-control	17,238	10,391	10,100	—	—	—

DISCUSSION

According to Lender (1960) decapitated planarians reared in water containing centrifugal supernatants of head homogenates either did not regenerate brains, or the brains were of reduced size. Lender felt that the diffusion of inhibitory substances from the brain gave rise to an anterior-posterior gradient of inhibition. In recent work on inhibitors Ziller-Sengel (1967a, 1967b) has reported that the pharyngeal region of the planarian *Dugesia lugubris* contains a specific inhibitor which delays the regeneration of the pharynx. The inhibitor is present in filtered extracts of the pharyngeal region and is species-specific since homogenates of the pharyngeal regions of *Dugesia tigrina* do not inhibit regeneration of the pharynx in *Dugesia lugubris*.

The results of this investigation do not demonstrate the presence of a specific inhibitor of brain regeneration in 10,000 × *g* supernatants of heads of *Dugesia dorotocephala*. Centrifugal supernatants of homogenates from the three body levels at concentrations of 2 to 87 cut sections in 2 milliliters of homogenization medium (6–600 μg protein/ml) do not exhibit inhibitory activity.

Brondsted (1955) showed that a head regenerates in a window cut out of the fore part of the planarian, *Bdellocephala*, when the old head is present. This implies that inhibitory substances from a certain body structure do not travel through the body to inhibit the regeneration of identical structures. Brondsted's experiment, and the reports of Ziller-Sengel (1967a, 1967b), showing a delay in the regeneration of the pharynx and not a loss or a reduction in size of the regenerated organ, do not support the role of specific inhibitors in maintaining polarity.

Exposure of posterior cut sections of planaria to nutrient media (chick embryo extract and chick embryo extract ultrafiltrate) after a sufficient time of starvation of the worms does alter the polarity of the regenerating pieces. It has been shown that cephalocaudal physiological gradients exist in planaria and that polarity of regenerates can be reversed by abolishing such gradients (Flickinger, 1959; Flickinger and Coward, 1962; Coward, 1968). Nutrient media (Coward, Flickinger and Garen, 1964) accelerate both the rate and extent of regeneration in posterior regions of cut starved planarians. Although very short cut pieces often form heads at both ends (Janus heads), this did not occur in the control pieces in saline. The levels of the worm just posterior to the pharynx (levels 5 and 6) are in the fission zone of the planarian and thus may account for bipolar head formation in this region.

It has been demonstrated that inhibition of DNA synthesis is necessary in order to produce bipolar head regeneration with cloramphenicol (Kohl and Flickinger, 1966). Therefore, it seemed important to learn if a stimulation of DNA synthesis, and presumably cell division, occurred when bipolar head formation is induced by stimulation, rather than by inhibition. The results of the H^3-thymidine incorporation experiments in which anterior, middle and posterior segments were regenerating in saline show that after 30 days of starvation there still exists a cephalocaudal gradient of DNA synthesis as described by Coward and Flickinger (1965), and Kohl and Flickinger (1966). However, no difference in DNA synthesis existed between the pooled middle and posterior regions. Furthermore, posterior body levels showed a greater increase in the percentage of stimulation of DNA synthesis with nutrient media than does the anterior level, indicating a greater stimulation of cell division in these levels than in the head level.

The results of this investigation, and the reported work of previous investigators, strongly suggest the presence of a cephalocaudal metabolic gradient in planarians in which metabolic competition results in the dominance of anterior levels over each succeeding posterior level. No evidence for specific inhibitor substances was found in this study.

SUMMARY

1. Decapitated planaria, *Dugesia dorotocephala,* exposed to head and tail centrifugal supernatants for nine days do not exhibit inhibition of brain regeneration. The experimental and control worms had brains of equal sizes.

2. A significant number of bipolar heads regenerated in small pieces from the posterior region of starved worms which were cultured in chick embryo extract and chick embryo extract ultrafiltrate.

3. Chick embryo extracts produce a stimulation of DNA synthesis in pieces of starved worms which were cut into anterior, middle and posterior sections. There is a greater stimulation at the posterior levels which can give rise to bipolar heads. This stimulation of DNA synthesis is thought to reflect an increase in cell division at these levels caused by the chick embryo extract.

LITERATURE CITED

BRONDSTED, H. V., 1955. Planarian regeneration. *Biol. Rev.,* **30**: 65–126.

CHILD, C. M., 1941. *Problems and Patterns of Development.* University of Chicago Press, Chicago, 727 pp.

COWARD, S. J., 1969. The relation of surface and volume to so-called physiological gradients in planaria. *Develop. Biol.,* **18**: 590–601.

COWARD, S. J., AND R. A. FLICKINGER, 1965. Axial patterns of protein and nucleic acid synthesis in intact and regenerating planaria. *Growth,* **29**: 151–163.

COWARD, S. J., R. A. FLICKINGER AND E. GAREN, 1964. The effect of nutrient media upon head frequency in regenerating planaria. *Biol. Bull.,* **126**: 345–353.

DISCHE, Z., 1955. Color reactions of the nucleic acid components. Pages 285–305 in E. Chargaff and J. Davidson, Eds., *The Nucleic Acids, Vol. 1.* Academic Press, New York.

FLICKINGER, R. A., 1959. A gradient of protein synthesis in planaria and reversal of axial polarity of regenerates. *Growth,* **23**: 251–271.

FLICKINGER, R. A., AND S. J. COWARD, 1962. The induction of cephalic differentiation of regenerating *Dugesia dorotocephala* in the presence of the normal head and in unwounded tails. *Develop. Biol.,* **5**: 179–204.

KOHL, D. M., AND R. A. FLICKINGER, 1966. The role of DNA synthesis in the determination of axial polarity of regenerating planarians. *Biol. Bull.,* **131**: 323–330.

LENDER, T. H., 1960. L'inhibition spécifique de la différentiation du cerveau des Planaires d'eau douce en regénération. *J. Embryol. Exp. Morphol.,* **8**: 291–301.

LOWRY, O. H., N. J. ROSEBROUGH, H. L. FARR AND R. J. RANDALL, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193**: 265–276.

MILLER, J. A., 1938. Studies on heteroplastic transplantation in triclads. I. Cephalic grafts between *Euplanaria dorotocephala* and *E. tigrina. Physiol. Zool.,* **11**: 214–247.

MORGAN, T. H., 1902. Growth and regeneration in *Planaria lugubrus. Wilhelm Roux Arch. Entwicklungsmech. Organismen,* **13**: 179–212.

NIU, M. C., AND V. C. TWITTY, 1953. The differentiation of gastrula ectoderm in medium conditioned by axial mesoderm. *Proc. Nat. Acad. Sci., Washington,* **39**: 985–989.

RAND, H. W., AND M. ELLIS, 1926. Inhibition of regeneration in two-headed or two-tailed planarians (maculata). *Proc. Nat. Acad. Sci., Washington,* **12**: 570–574.

ZILLER-SENGEL, C., 1967a. Recherches sur l'inhibition de la régénération du pharynx chez les planaires. I. Mise en évidence d'un facteur auto-inhibiteur de la regeneration du pharynx. *J. Embryol. Exp. Morphol.,* **18**: 91–105.

ZILLER-SENGEL, C., 1967b. Recherches sur l'inhibition de la régénération du pharynx chez les planaires. II. Variations d'intensite du facteur inhibiteur suivant les espèces et les phases de la régénération. *J. Embryol. Exp. Morphol.,* **18**: 107–119.

Reference: *Biol. Bull.*, **140**: 125–136. (February, 1971)

FINE STRUCTURE AND COMPOSITION OF A SILICEOUS SPONGE SPICULE[1]

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When a siliceous skeletal spicule of a marine sponge is dissolved in hydrofluoric acid (HF), a filament remains. We report here a new understanding of the structure and composition of the siliceous spicule and its axial filament. Bütschli (1901) showed that these filaments stain with several organic dyes and behave toward a variety of reagents as protein. He concluded that the HF-resistant residue, presumably all axial filament, contained a very small amount of organic material around which silica deposition is initiated. The view that the *whole* axial filament, or even a major fraction of it, is "sans doute protéinique" (page 497 Lévi, 1963) appears to be a misreading of this statement by Bütschli. Bütschli further showed that spicules fractured in cross section and stained with various dyes exhibit a triangular core and several regularly spaced concentric rings. The laminar structure has been widely documented for large spicules. The early literature is exhaustively reviewed by Minchin (1909).

Drum (1968) applied rotary replication techniques in the study of the surface ultrastructure of siliceous spicules. In his electron micrographs he points out a filament that remains after HF removal of the spicule. From its binding of dyes and its loss on ultramicroincineration he concluded that the filament was an organic axial filament, primarily carbohydrate. He did not obtain enough material for direct chemical analysis.

We present three kinds of data on the structure and composition of the axial filament and the surrounding siliceous spicule. These are direct chemical analysis, phase-contrast light micrographs, and electron micrographs obtained by both direct (rotary shadow) and indirect (negative replica) procedures. Our major conclusion is that there is sufficient carbon in the siliceous spicule to provide a major fraction of the visible axial filament as a protein or carbohydrate. Nevertheless, organic matter is only a small fraction of the HF-resistant residue. This conclusion is not contrary to that of Bütschli, but is contrary to that of more recent workers who have cited Bütschli as their authority. The analytical methods used here were not available to Bütschli and have not been combined in this manner by others.

MATERIALS AND METHODS

Growth and maintenance of sponges

Clumps of marine sponge were obtained through Pacific Biomarine from the coast of California near Los Angeles. These were held with sea urchins

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and other marine animals in a recirculating aquarium (Instant Ocean) at 13° in artificial sea water (Instant Ocean) to which has been added sodium silicate to give 1 m*M* silicate. Growth of new tissue occurred in this aquarium. The sponge was identified as to species on the basis of morphology and spicule type. *Acarnus erithacus* has a peculiar acanthocladotyle as a minor macrosclere (DeLaubenfels, 1932). The spicules studied were the major macrosclere, a simple style.

Preparation of spicules

Spicules were freed from the surrounding sponge tissue and other contaminants in several ways: (a) nitric acid and density gradient centrifugation, (b) nitric acid and water washing, (c) crude enzymatic digestion and water washing. For light microscopy and some of the chemical analysis, spicules were freed from the sponge tissue by digestion in boiling concentrated nitric acid. The acid was washed off by repeated centrifugation through distilled water. The spicules were separated from organic debris and sand by isopycnic centrifugation on a density gradient of carbon tetrachloride and ethylene dibromide. They were washed in methanol and dried at 20°. For electron microscopy spicules were cleaned in concentrated nitric acid at room temperature (4 hours) and washed in distilled water. Finally the styles were separated from the other spicules by differential settling in distilled water and air dried at 60°.

Chemical analysis

Acid-washed, density gradient spicules were freed from tiny flakes (presumably mica) of the same density (1.93 to 1.96) by gentle swirling in a shallow dish. The spicules were dried from methanol in a tared Teflon weigh boat. The spicule cake was flooded with 1 *N* HF and held at 55° on a water bath to dryness. This was repeated to constant weight of the residue at 55°. Portions of the weighed, air-dried residue were subjected to three different analyses: (a) gas chromatography for C, H, and N, (b) emission spectrography, and (c) atomic absorption.

Light microscopy

Nitric acid-washed spicules were observed by phase-contrast light microscopy in a Wild M20 research microscope. The photographs in this paper were made with a 100 × oil immersion objective on Kodak Panatomic-X 35 mm film. Spicules were placed between a plastic cover glass and a plastic dish and individual spicules observed from the time the HF reached them. Timed observations thus represent time after contact of the observed spicule with the etching fluid. A spicule and its remnants were observed for as long as 2 hours.

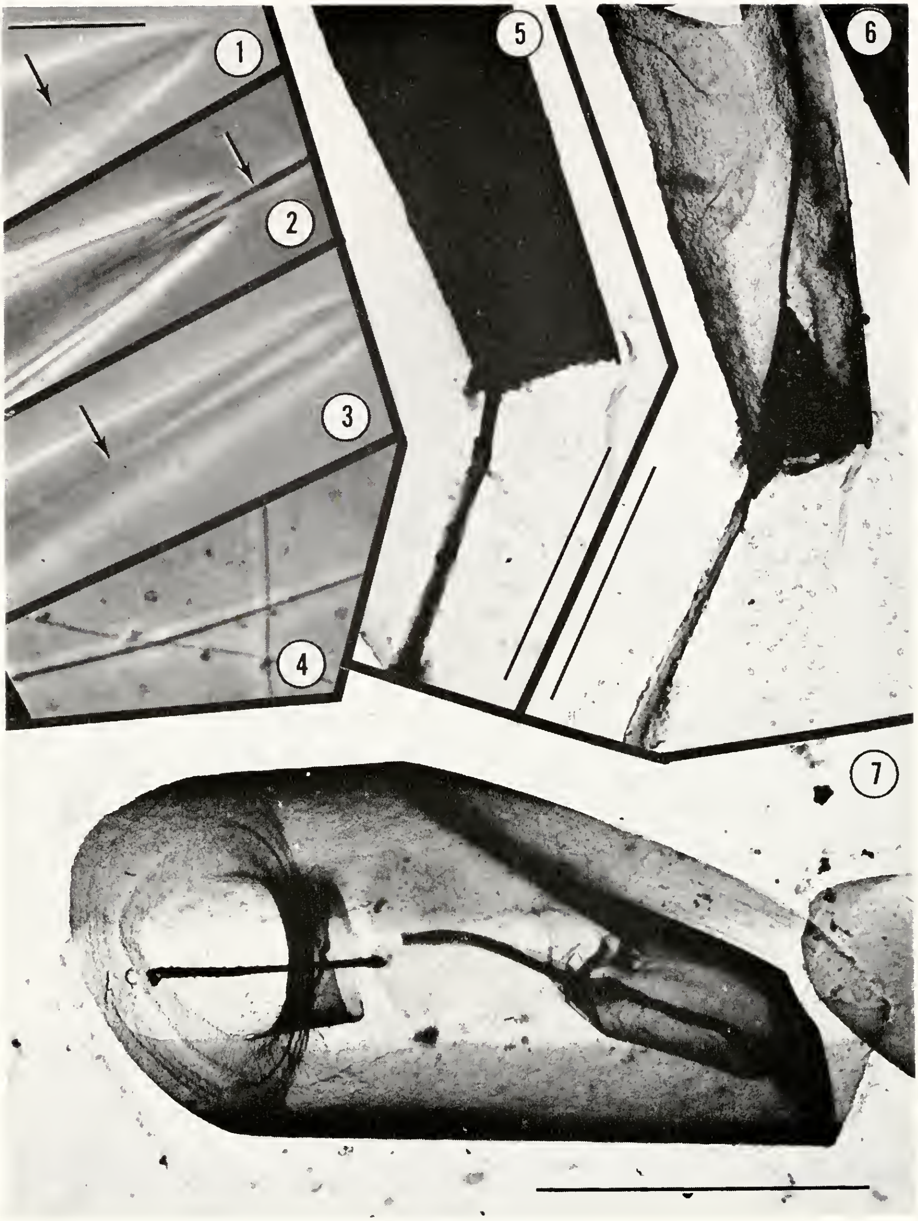
Electron microscopy

Drum (1968) developed a procedure using rotary shadow to make a replica from which the spicule was then removed by HF digestion. This three-dimensional replica allowed him to study the surface well, but the internal filament was often displaced or obscured. The technique was modified in our laboratory to facilitate study of the filament. The spicule was partially removed by a brief etch in HF, exposing a portion of the axial filament. After rotary shadowing, the remaining spicule was removed by a second digestion in HF. The axial filament was held attached to the replica. Cleaned spicules were broken with the edge of a small spatula and sprinkled loosely onto a collodion-coated 200 mesh electron microscope grid. One drop of aqueous 5% HF was placed on the gird. After 2-½ to 3 minutes the drop was removed by touching the edge of the grid with filter paper. In a similar manner the grid was washed in three changes of distilled water and dried at room temperature. A carbon film was evaporated *in vacuo* onto the specimen during two to three complete revolutions of the sample. The remaining spicule was removed by floating the grid, spicule side down, on a drop of HF for 15 minutes. The grid was again washed three times in distilled water in the same manner.

TABLE I

Chemical analysis of spicules cleaned in boiling HNO_3

Component	Weight as % of spicule	Weight as % of residue	Atomic ratio to Si in residue (×100)
Part A: By loss of weight in 1 *N* HF at 55°			
$SiO_2 + H_2O$	95.7		
Residue	4.3	100.0	
Part B: By atomic absorption, expressed as the element determined			
Si	—	17.5	100.0
Na	0.65	13.7	94.0
K	0.124	2.57	10.5
Al	0.154	3.07	18.2
Ca	0.014	0.29	1.16
Fe	0.007	0.14	0.4
Part C: By combustion and gas chromatography of the oxide, expressed as the element determined			
N	0.073–0.57	1.46–11.5	
H	0.022–0.08	0.43–1.6	
C	0.016–0.042	0.32–0.83	
Part D: By semi-quantitative emission spectrography, expressed as the oxide of the element named			
B		0.6–6.0	
Ba		0.03–0.3	
Mg, Ni, Ti, Zr (each)		0.02–0.2	
Pb		0.01–0.1	
Sr		0.006–0.06	



FIGURES 1–7

FIGURES 1–4. Phase contrast photomicrographs of spicules and axial filaments in HF during digestion, all same magnification, scale line = 20 μ.

FIGURE 1. Intact spicule at first contact with HF showing axial filament (arrow).

FIGURE 2. Digestion in 1 *N* HF alone leaves the axial filament (arrow) protruding from partially digested siliceous cylinder.

A technique was also devised for studying the ultrastructure of spicules in cross section. In general terms, lightly etched spicule sections were replicated with cellulose acetate. The replicas were removed and shadowed, and this negative image examined. Cleaned spicules were embeddd in Epon 812 and polymerized at 60° for 48 hours. The blocks were cut with a diamond saw so as to expose cross sections of a maximum number of spicules. The sawed surface was polished with Barnesite (Edmund Scientific Company) polishing compound and cleaned in an ultrasonic cleaner with distilled water. A negative replica of the polished surface was made by evaporating a drop of 8% cellulose acetate in acetone on the surface. After about 4–6 hours, the dry plastic negative replica was stripped off. The replica was shadowed with Pt-C ($\tan^{-1} \frac{1}{3}$) and carbon coated. Spicule-containing areas were cut out, placed on 200 mesh electron microscope grids, and the plastic dissolved with acetone in a vapor condensation washer. The embedded spicules were then etched for 30 seconds in 5% HF and replicated again as before. Multiple replicas were made of the same surface. Specimens were studied in a Siemens Elmiskop I at 80 KV accelerating voltage.

Observations

Chemical analysis

The results of the three types of chemical analyses are all summarized in Table I. The first column shows the composition for the whole spicules, the second for the non-volatile residue after 1 *N* HF digestion. The table compares the amounts of the various major elemental components on the basis of weight as a percentage of total spicule (column 1), weight as a percentage of the non-volatile residue after HF (column 2), and atoms per atom of silicon in the residue (column 3). The spicules would appear to be primarily silicic acid (or silica, since the H_2O content was not directly determined). The residue may contain alkali silicates, but to have all the sodium and potassium present as silicate would require more silicon than is found. Were there very little alkali silicates in the residue there would be sufficient silicon for all the other cations to be present as metal silicates. The proportion C:H:N in the residue is between 1:50:6 and 1:100:260 as calculated from the range of values from the two portions of the inhomogeneous residue. In both cases the total carbon is in the range of 1% of the residue; therefore, less than 2% of the residue could be organic matter of the 40% C composition expected of proteins and carbohydrates. Since the N:H ratio is so high it seems unlikely that the N is present as an amine, but more probably as a nitrate.

FIGURE 3. Digestion in 1 *N* HF in the presence of citric acid removes the axial filament. The axial filament may be preferentially removed deep into the siliceous cylinder; arrow marks retreating tip of axial filament. Compare to Figure 1.

FIGURE 4. After completed digestion in HF, axial filaments remain (3 shown).

FIGURE 5. Electron micrograph of a spicule after partial removal in HF showing an axial filament protruding from the end, scale line = 10 μ.

FIGURE 6. Same spicule as in Figure 5 after rotary replication and complete removal in HF. The axial filament can be seen extending from the tip of the conical etch pit, scale line = 10 μ.

FIGURE 7. Spicule fragment partially removed in HF, rotary shadowed, and then completely removed in HF. The etch pit is only partially replicated but the axial filament is evident, scale line = 10 μ.

Light microscopy

Measurements with an eyepiece micrometer agree with the published dimensions for the styles of *Acarnus erithacus,* namely $20\mu \times 340\mu$.

An axial structure can be seen inside intact spicules at high magnification with phase optics. Figure 1 is a photomicrograph showing such a structure. It does not appear to be smooth but angular in profile. It does appear to be cylindrically symmetrical. During digestion in 1 *N* or 2 *N* HF, the siliceous outer portion of the spicule is removed and the axial filament remains (Figs. 2 and 4). The axial filament may be observed to kink, wave in the fluid, or lie on the substratum. Prolonged exposure to 2 *N* HF does not visibly alter the filament.

By contrast to the persistence of the filament in HF alone, when citric acid is added to the HF (from 2 to 4.8 *M* citric) both the outer and axial portions of the spicule vanish within 15 minutes. The axial filament may even be destroyed prior to the siliceous cylinder. This is illustrated by a comparison of Figure 2 (HF alone) and Figure 3 (HF and citric). The siliceous outer portion of the spicules is at about the same stage of digestion.

Electron microscopy of whole spicules

With the rotary shadow technique it was possible to study the axial filament from a spicule that had been partially removed, and then to remove the HF-sensitive material completely and study the same region again by examining the carbon replica made during the rotary shadowing. Figure 5 shows a partially removed spicule with a filament extending from the end. The eccentric position of the filament will be understood after comparison with Figure 6, which is a replica of the *same spicule* seen in Figure 5 after the spicule was completely removed. The internal relationship of the filament and spicule is revealed. The removal of siliceous material by HF occurs most rapidly in the interior of the spicule, producing a conical etch pit. The filament can be seen to lie along the face of this cone extending outside the spicule from the edge of the cone's base and extending inside the spicule from the cone's apex. The carbon of the replica apparently is attached to the filament holding it in position as it was prior to removal of the second portion of spicule. For deep etch pits at unfavorable orientations to the carbon during replica formation, or for thin carbon coats, the filament may not be attached to the replica on the surface of the spicule. As Figure 7 shows, the interior portion of the filament is displaced slightly in such circumstances.

Electron microscopy of spicule cross sections

These figures are derived by shadowing a *negative replica* of the surface of the spicules so that protrusions on the spicule become pits on the replica, and pits on the spicule are protrusions on the replica. The conical etch pit already seen in the whole mount (positive shadow replica) is here a conical protrusion with a long shadow free of platinum. The etching time for the embedded spicules was necessarily shorter than for the partial removal of whole spicules. The result is much smaller etch pits and finer detail than was seen with the whole-spicule preparations.

Figure 8 is a cross section of a style, the major macrosclere of *Acarnus erithacus* that was polished but not etched. Similar preparations after light etching with HF are shown in Figures 9 and 10. Occasionally, the axial filament

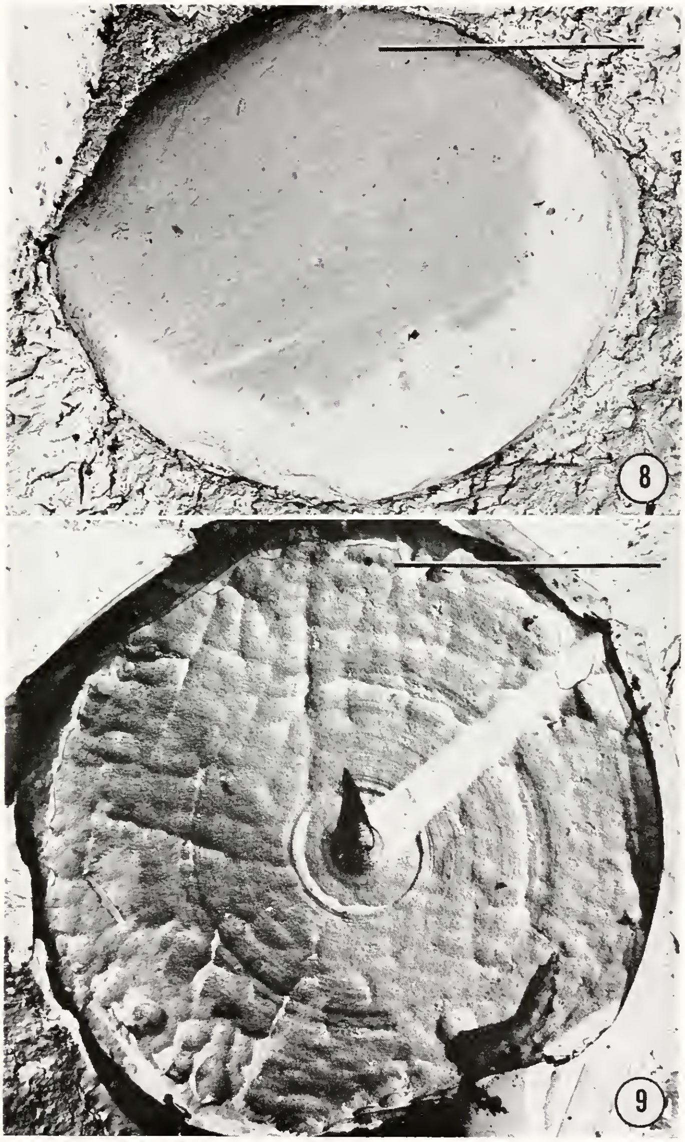


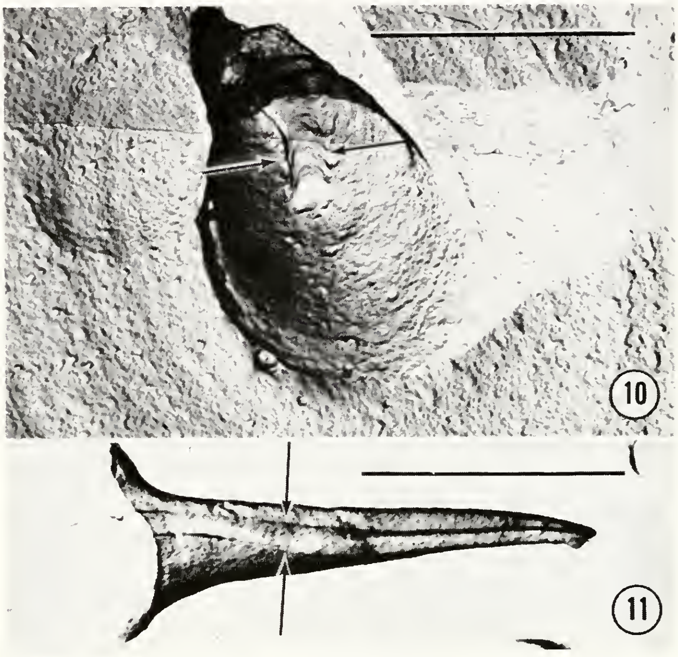
Fig. 8–9

Figure 8. Electron micrograph of a spicule cross section after polishing but no etch; C/Pt shadowed negative replica, scale line = 10 μ.

Figure 9. Spicule cross section after polishing and a 30-second etch in 5% HF. The center etches very rapidly and forms a conical etch pit. Closely spaced concentric rings are also evident; C/Pt shadowed negative replica, scale line = 10 μ.

may be seen as a detail in the etch-pit cone (Fig. 10). Oblique sections through spicules provided supplementary evidence of the axial position of this filament, as shown in Figure 11. The shape of the axial filament is not clear from these observations. It may be either triangular or circular in section. The filament appears to be about 0.4–0.6 μ in diameter.

The negative replicas exhibit circular surface protrusions (Figs. 9 and 10), indicating the existence of indentations produced by etching on the surface of the spicule. The more pronounced, that is, deeper, rings may correspond to those described by Bütschli (1901) from light microscopy of stained fractured spicules. Several shallower grooves occur between and concentric with the deeper clefts. The rings are usually more pronounced closer to the center of the spicule. This is correlated with the more rapid removal of material in this region during etching that is expressed as the conical etch pit already mentioned above. The rings are somewhat irregularly spaced. The thinnest ones are about 0.2–0.3 μ thick.



FIGURES 10–11

FIGURE 10. Replica of an axial filament in an etch pit (arrows); C/Pt shadowed negative replica, scale line = 1 μ.

FIGURE 11. Replica of an etch pit in oblique section showing the axial filament (arrows); C/Pt shadowed negative replica, scale line = 1 μ.

DISCUSSION

The pattern of chemical composition revealed by our analysis is in general agreement with the literature. The bulk of the siliceous spicule is cation-free oxide of silicon, presumably $(SiO_2)_xH_2O$. The present data provide no value for the ratio of SiO_2 to water. Values have been reported in the literature ranging from 3 to 5. (Minchin reviews the literature before 1909; see also Jørgensen, 1944).

There is little agreement in the literature on the cation composition of siliceous spicules, presumably reflecting real differences among the forms examined (Minchin, 1909). To compare two marine Demospongiae, the present values are in agreement with Bütschli's for sodium and the traces of aluminum, though he found much more magnesium.

From the composition and the density there is no reason to question the use of the term "spicopal" to refer to the siliceous portion of the spicule (Vosmaer and Wijsman, 1905), that is, to presume an amorphous silicic acid with traces of various cations with properties similar to the mineral opal.

As to the nature of the axial filament, there can be little question now that the siliceous macroscleres have a component part that is both axial and HF-resistant. The filament can be directly observed before and during etching. The high magnification light microscopy leads us to conclude that the filament visible by phase microscopy is not derived from the outer surface of the spicule by collapse of a net or sheath. Nor can we see how it could be derived by the coalescence of isolated particles within the siliceous matrix that come together during dissolution of the spicule. Organic matter on the native spicule surface would presumably have been destroyed by the 4-hour digestion in boiling concentrated nitric acid. The direct observation during solution of the spicule in HF reveals an object protruding from the end of the retreating spicule that has the same dimensions as a line found within the spicule prior to etch.

What portion of the axial filament may be protein or other organic material? The destruction of axial filament in mixed citric-hydrofluoric acid indicates only that a chelatable cation contributes to the stability of the filament. Other similar organic substances have been characterized; in particular a presumably unrelated factor involved in cell aggregation has been purified from sponge (Moscona, 1968). Its insolubility depends on the presence of Ca^{++} or other divalent cations. The most direct evidence of the possibility of a primarily organic filament comes from the CHN analysis. The carbon present (between 0.32 and 0.83% of the residue) would be sufficient to account for at least 0.8% of the residue as organic matter with 40% carbon, the range found in carbohydrates and proteins. As a fraction of the total spicule weight, this would be at least 4.0×10^{-4}. The filament visible in electron micrographs appears to be about 0.5 μ in diameter and the spicules are about 20 μ in diameter. The ratio of filament cross section to spicule cross section, that is, the ratio of radii squared, has a value of 6.3×10^{-4}. Mass density of the organic material would be expected to be around 1.27 (gelatin) to 1.53 (starch) and of the spicopal about 1.96 (whole spicules) to 2.3 (opal). Thus a given mass of organic material would fill about 1.5 times the volume of the same mass of opal.

When the carbon found (expressed as the organic fraction of the total spicule), 4.0×10^{-4}, is multiplied by this mass density correlation factor, 1.5, the product is 6.0×10^{-4}. That is, organic matter makes up by weight 0.4 parts per thousand of the whole spicule, and that much is expected to occupy 0.6 parts per thousand by volume. We observe a structure that occupies about 0.63 parts per thousand by volume. We are led to conclude that there is sufficient carbon detected to provide a major fraction of the visible axial filament as organic matter with the carbon content of protein or carbohydrate. It is important to emphasize that this must be a minimum estimate since the harsh cleaning procedure, boiling concentrated nitric acid, while removing the organic material from the native surface of the spicule might penetrate into the open tip of some spicules and remove some material.

The data of Travis, François, Bonar, and Glimcher (1967) deserve comment at this point. As part of a broad comparative study of the organic material associated with the mineralized skeletons in animals, they examined the amino acid composition of an HF residue of siliceous spicules. They cleaned the spicules with distilled water only. Such a treatment is not likely to remove the collagen fibers that bind spicules in place, to say nothing of less specific cellular debris. Therefore, their data apply to a mixture of surface and axial material. It may well be that the surface material is more important in controlling the actual deposition of the siliceous body of the spicule.

A second recent paper of importance is by Drum (1968). His conclusions on organic material rest primarily on incineration data. Unfortunately, organic material is not the only material volatilized by such a procedure.

All three microscopy techniques used in this study indicate the existence of an HF-resistant filament that is axial in position. From the chemical analysis there would seem to be sufficient organic matter to account for the filament as carbohydrate or protein. Organic axial filaments have been demonstrated in calcareous spicules (Minchin and Reid, 1908). Jones (1967) refutes much of that work, contending that the filaments are preparation artifacts and at best are impure calcite in the intact spicule. The present work does not appear to be subject to that kind of criticism.

Concentric rings are revealed by gentle etching of spicule cross sections. From fracture studies the earlier workers, reviewed by Minchin (1909), had concluded that the spicule was composed of concentric lamellae. Schulze (1904) observed a similar ring pattern in Hexactinellids which he interpreted as lamellae of organic matter alternating with siliceous material.

According to Bütschli, the concentric strata were layers of different refractility. The lowered refractility, he surmised, arose from a minutely alveolar structure. Our figures show that some of the rings are clefts and some are dikes, thus differing in etch sensitivity. It is not clear how such a pattern of concentric but irregularly spaced zones of etch-resistant and etch-sensitive material could be related to Schulze's pattern of regular rings, presumably of organic material. Bütschli's notion of differences in compactness appears to be clearly related to the kind of pattern we see. However, if microalveoli are present, they must be below the resolution of our replica technique.

We must further emphasize the discrepancy in scale of the parts of the 20-μ diameter spicule as reported here for *Acarnus* and as reported by Bütschli for *Tethya* and *Geodea*. His axial filament is about 2.4 μ in diameter, that is, a fraction 0.12 of spicule diameter. His concentric rings are about 1.8 μ thick, that is, 11 rings for the entire 20-μ diameter spicule. Our filament diameter is about one-fourth of his, both in absolute terms and as a fraction of spicule diameter. Our rings are similarly smaller than his by a factor of 0.14. Rings of this small size are at the limit of resolution of the light microscope. It is easily understandable that Bütschli would not have detected these as individual rings.

We cannot rule out the attractive possibility that subtle differences in composition or physical structure of the spicopal give rise to both the differences in etch sensitivity that we report and the negative birefringence that others have reported (Minchin, 1909).

Investigations are underway in this laboratory to determine the spatial distribution of chemical elements within the spicule.

We gratefully acknowledge the expert assistance of several colleagues for providing chemical analyses; W. H. Grieve for gas chromatography, R. H. Hall for emission spectrography and T. J. Gomoll for atomic absorption.

SUMMARY

Siliceous spicules from *Acarnus erithacus* were studied using electron and light microscopy in conjunction with chemical analyses by gas chromatography, emission spectrography, and atomic absorption. Chemical data correlated with microscopy measurements indicate that there is sufficient carbon to provide a major fraction of the axial filaments as organic matter (assuming 40% carbon). The concentric ring structure reported earlier from light microscopy was studied on the electron microscopic level by utilizing carbon-platinum replicas of HF etched spicule cross sections. Ring spacings as small as 0.2–0.3 μ were detected using this technique. Correlation of chemical composition and fine structure is discussed with respect to the axial thread and the siliceous portions of the spicule.

LITERATURE CITED

BÜTSCHLI, O., 1901. Einige Beobachtungen über Kiesel- und Kalknadeln von Spongien. *Z. Wiss. Zool.*, **69**: 235–286.

DELAUBENFELS, M. W., 1932. The marine and fresh-water sponges of California. *Proc. U. S. Nat. Mus.*, **81**: 1–140.

DRUM, R. W., 1968. Electron microscopy of siliceous spicules from the freshwater sponge *Heteromyenia*. *J. Ultrastruct. Res.*, **22**: 12–21.

GARRONE, R., 1969. Collagène, spongine et squelette minéral chez l'éponge *Haliclona rosea* (O.S.) (Démosponge, Haploscléride). *J. Microscop.* **8**: 581–598.

JONES, W. C., 1967. Sheath and axial filament of calcareous sponge spicules. *Nature*, **214**: 365–368.

JØRGENSEN, C. B., 1944. On the spicule-formation of *Spongilla lacustris* (L.) 1. The dependence of the spicule-formation on the content of dissolved and solid silicic acid of the milieu. *Det. Kgl. Danske Videnskabernes Selskab Biologiske Meddelelser*, **19**(7): 1–45.

LÉVI, C., 1963. Scléroblastes et spiculogenèse chez une éponge siliceuse. *C. R. Acad. Sci. Paris*, **256**: 497–498.

MINCHIN, E. A., 1909. Sponge-spicules. A summary of present knowledge. *Ergebnisse und Fortschritte der Zoologie*, **2**: 171–274.

MINCHIN, E. A., AND D. J. REID, 1908. Observations on the minute structure of the spicules of calcareous sponges. *Proc. Zool. Soc. London,* **2**: 661–676.

MOSCONA, A. A., 1968. Cell aggregation: Properties of specific cell-ligands and their role in the formation of multicellular systems. *Develop. Biol.,* **18**: 250–277.

SCHULZE, F. E., 1904. Hexactinellida. [*Wissenschaftliche Ergebnisse der Deutschen Tiefsee-Expedition auf dem Dampfer "Valdivia" 1898–1899,* Vol. **4**] G. Fischer, Jena, 266 pp.

TRAVIS, D. F., C. J. FRANÇOIS, L. C. BONAR AND M. J. GLIMCHER, 1967. Comparative studies of the organic matrices of invertebrate mineralized tissues. *J. Ultrastruct. Res.,* **18**: 519–550.

VOSMAER, G. C. J., AND H. P. WIJSMAN, 1905. On the structure of some siliceous spicules of sponges. I. The styli of *Tethya lyncurium. Acad. Wet. Amsterdam, Proc.,* **8**: 15–28.

Reference: *Biol. Bull.*, **140**: 137–155. (February, 1971)

PHOTOPERIOD CONTROL OF DIAPAUSE IN *DAPHNIA*. IV. LIGHT AND CO_2-SENSITIVE PHASES WITHIN THE CYCLE OF ACTIVATION

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The continual involvement of photoperiod in the life cycle of arthropods is demonstrated by the influence of photoperiod on both the initiation and termination of diapause (Adkisson, 1965). Photoperiod control of diapause in insects has been demonstrated for larval (Paris and Jenner, 1959; Wellso and Adkisson, 1964), pupal (Williams and Adkisson, 1964) and imaginal stages (deWilde, Duintjer and Mook, 1959). Embryonic diapause is also known to be initiated in response to photoperiodic induction. This has been demonstrated for a number of insects and for the Crustacean, *Daphnia pulex* (Stross and Hill, 1968).

In previous study with *Daphnia* the termination of diapause may require a light stimulus (Stross, 1966, 1969), thereby demonstrating the presence of a photoreceptor which may be absent in early embryos of some insects (Minis and Pittendrigh, 1968). Furthermore, the light stimulus may need to be "long-day" although many embryos are activated in "short-days" as well. Since photoperiod control of diapause initiation may be controlled by density of the experimental animals, *i.e.,* facultative, the suggestion is that an unknown stimulus present in the water may interfere with the retention of diapause in short daylengths. The present study was undertaken to demonstrate photoperiod control of diapause termination in *Daphnia.*

A second objective of the study was to test the applicability of two empirical models of photoperiodism, a test made possible by the remarkable feature of the diapaused embryo of *Daphnia* to respond to a single light period. Bunning (1959, 1964) described a physiological rhythm of maximum light sensitivity, the timing of which he suggested may be more influenced by the "dawn" than by the "sunset" of a natural or simulated day. A restatement of the model was made possible by a more explicit knowledge of the action of light on a circadian rhythm in an insect. The restated (coincidence) model (Pittendrigh and Minis, 1964) argues that the so-called time or phase of maximum sensitivity to light (photo-inducible phase) is coupled to the circadian cycle of the organism. Thus the light period of a daily cycle phases the circadian cycle, and when the light period is "long-day," it illuminates the organism at a time when it is photo-inducible. The dual effect of light is operationally significant when the experimental organism requires more than one inductive cycle. The coincidence model is satisfied when the long-day response is induced by a single pulse of light per cycle administered to an organism at the photo-inducible phase of its circadian cycle. The position of the inducible phase was first indicated to be near sunset (Pittendrigh and Minis, 1964) and later generalized to include the possibility that it could be at dawn (Pittendrigh, 1966).

An alternative model of photoperiodism (Lees, 1966) suggests that time measurement is restricted to the dark period, and a long-day condition results (virginoparae) when the night is critically short (Lees, 1966). In an organism requiring but a single period of light for induction, two pulses of light suitably spaced with respect to one another but independent of the organism's circadian cycle would suffice to induce the long-day condition. This so-called "interval-timer" has been tested with consistent results in the aphid *Megoura*. A recent study (Hamner, 1969) with a moth (*Carpocapsa*) strongly indicates the presence of both a circadian and an interval-timer in the photoperiodic response.

Previous study has shown that the diapaused embryo of *Daphnia* contains a minimum of two phases, a photo-refractory phase followed by a photo-sensitive phase (Stross, 1965). The photo-refractory phase in embryos collected from the wild is completed with exposure to low temperature; 4° C was found adequate for embryos collected at temperate latitudes (Stross, 1966, 1969) but a lower temperature was necessary for embryos of *D. middendorffiana* collected from the arctic (unpublished). The duration of the photo-refractory phase may be different in summer and winter diapausing strains, and much shorter in the former (Stross, 1969). The alternative condition of having the same or similar thermal optimum for both the active and diapause states has also been shown to exist in Supply House cultures of the species (Stross, 1966; Davison, 1969).

The photo-sensitive phase in *Daphnia pulex* may or may not require light for terminating the embryonic diapause. In a winter diapausing strain, a light requirement is restricted to two situations: pre-mature termination in autumn before the diapause state intensifies, and in the spring when the embryos are incubated in a crowded and presumably oxygen deficient environment (Stross and Hill, 1968). In other strains including the ones lacking a low temperature optimum, light remains an absolute requirement, that is the diapause is maintained indefinitely in constant darkness. The suggestion that the process underlying activation may be basically photoperiodic has been apparent in both light-escaping and light-requiring strains (Stross and Hill, 1968; Stross, 1969).

METHODS AND MATERIALS

The egg pods (ephippia) containing usually a pair of diapaused embryos were removed from the bottom of the culture vessel and transferred to constant dark. They were mass incubated while either lying at the bottom of beakers (stagnant environment) covered with saran or suspended in nylon net within a flowing stream (Fig. 1). The embryos were mass produced in cultures of a strain of *Daphna pulex* Leydig that came originally (1960) from a Biological Supply House. The strain is capable of completing the photo-refractory phase at 20° C (Stross, 1966), the incubation temperature in these experiments, although the environment may require other modification (see Results). The strain could be classed as the dicyclic type since at room temperature (21°) the females readily reproduce exclusively the diapausing embryos when densely cultured in long day-lengths. The cultures were maintained in room light supplemented with fluorescent lighting on an L18:D6 regimen.

At the time of light exposure embryos were transferred under safelight to individual vials (25 × 95 mm) containing 20 ml of medium which was either lake water or a synthetic substitute (Stross and Hill, 1968). Ten or 25 egg pods containing a determined number of embryos were placed into each vial. The vials were then covered with saran or, as in the gasing experiments, stoppered with a rubber stopper lined with saran. Vials were prepared by autoclaving in a strong bicarbonate solution followed by rinsing in distilled water; this treatment was found to reduce the variance which can be a serious problem in hatching experiments. The embryos were exposed to "cool-white" fluorescent light and at intensities ranging from 1000 to 2000 lux, depending on the experiment, for intervals as indicated.

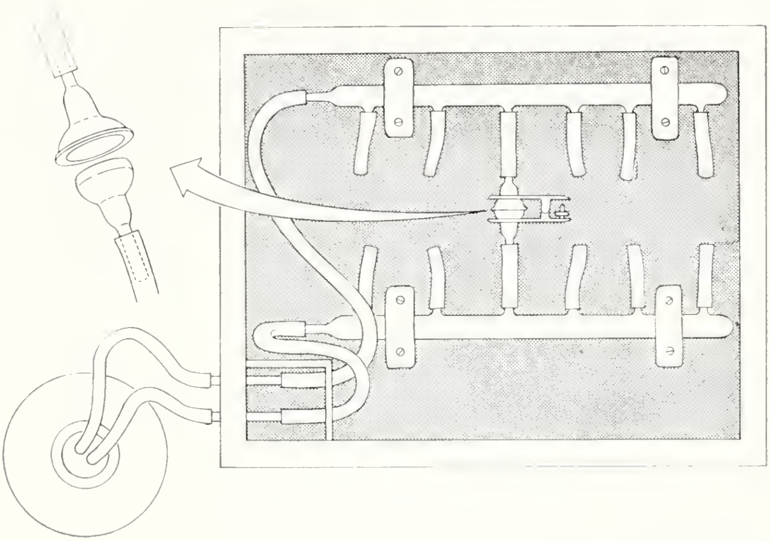

FIGURE 1. A "flowing stream" for incubating diapaused embryos of *Daphnia* in constant darkness. The embryos are held in small cylinders of nylon screen which is inserted into the ball joints between the manifolds.

In the experiments with modified atmosphere, the gas was introduced from prepared compressed sources (Linde-Union Carbide) with a sintered glass diffuser. The diffuser was introduced directly into the vial and the gas bubbled for 1.0 minute. Enriched CO_2 mixtures consisted of 10.0 and 49.0 per cent CO_2, a 20 per cent O_2 concentration, and the balance N_2. A 5.0 per cent CO_2 atmosphere was also used in which the CO_2 had been added to air (*i.e.*, displacing both O_2 and N_2). Enrichment with CO_2 was carried out at the time of light exposure. Preliminary treatment with N_2 was carried out in the same manner, except that masses of embryos were treated in screw-top jars which were sealed following gasing. This procedure may not have removed all O_2 from the medium, although in the CO_2 enrichment the treatment was sufficient to bring the medium to a new pH equilibrium.

Results

Activation of the diapaused embryo of *Daphnia pulex* Leydig may require light received as one long day or its "skeleton" (Fig. 2). In two experiments, light exposure was necessary at two separate intervals within a single light period. Embryos which had been incubated in constant dark and a natural thermocycle were first exposed to light at 0900 EST either for 2 or 16 hours. Those exposed for only two hours required a second exposure and the timing of the second exposure was critical. When exposed to a second pulse from hours 14 to 16 following the start of the first pulse, activation (63.6 per cent), as measured by hatching, was equivalent to a continuous 16-hour light exposure (72.0 per cent). A second exposure from hours 10 to 12 was not effective (0.0 per cent hatch) as was a single exposure given from hours 14 to 16.

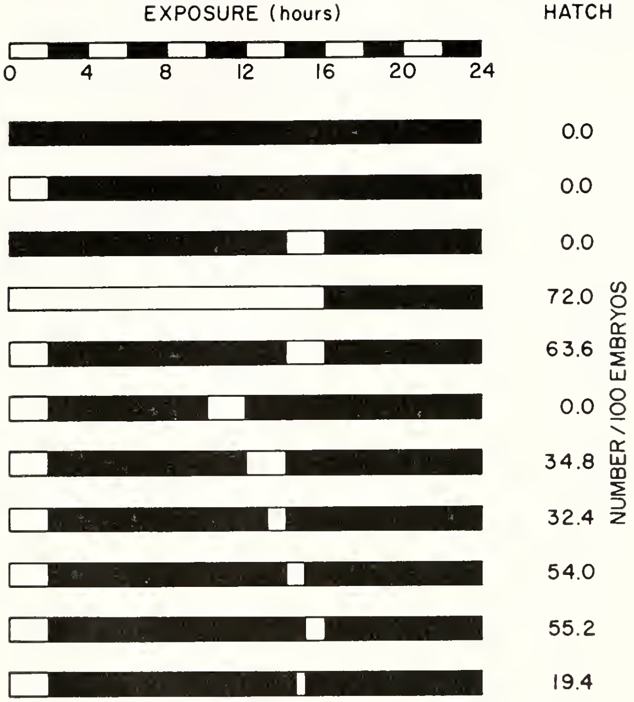

FIGURE 2. Hatching of *Daphnia* embryos when given a single 16-hour light period or two pulses of light that form the skeleton of one 16-hour light period. The embryos were first exposed at 0900 EST after having been in constant darkness and near an open (September) window for 17 or 31 days. Prior to that time the embryos were in constant darkness and temperature (19° C) for four months. (See text for additional details.)

Exposure to one 14-hour skeleton resulted in the hatching of ½ (34.8) the number activated by one 16-hour skeleton. This result suggested that the critical photoperiod for the embryos was approximately 14 hours of light. Further deductive support for 14 hours as the critical daylength was given by the number of embryos activated with a light pulse of shorter duration than the standard two hours. A one-hour pulse from hours 13 to 14 activated as many embryos as the standard pulse given from hours 12 to 14, whereas a one-hour pulse given after hour 14 resulted in the activation of a larger number of embryos. Further reduction in the duration of the light pulse, as for example to one-half hour, reduced activation (Fig. 2) and verified results of preliminary experiments. It is to be emphasized that these results were obtained from exposure of the embryos to a single photoperiod or its skeleton.

The requirement for two exposures appropriately spaced suggested that two photo-inducible phases exist and exposure to both may be a necessary part of long-day induction in *Daphnia* embryos. Lees (1966) has also shown that two exposures per 24 hours were necessary to simulate long daylength in the aphid, *Megoura,* or more precisely, to keep the night interval critically short. The apparent similarity between the two evoked such questions as whether the day or night interval was being measured and whether the position of at least one of the photo-inducible phases recurred at circadian intervals. Further experimentation proved impossible, however.

The long day or two-pulse requirement proved transitory for embryos held in the dark in a stagnant environment. Subsequent tests with the same batch of embryos, but from different containers, revealed first the loss of the requirement for a second pulse followed by a de-synchronization of photo-sensitivity. In the third experiment, designed to locate the time of photo-sensitivity relative to the thermal dawn of the environment, the first two-hour pulse of light activated as many embryos as the skeleton of a 12 or 16-hour photoperiod. In this new state the embryos were not only responsive to a single pulse but showed a time dependent sensitivity to photo-activation. Three groups of embryos were exposed at the onset of thermal rise (0600) in an artificial thermoperiod, or before (0400) or after (1000) the thermal rise. Activation, as measured by hatching, was largest in the embryos exposed to light at 1000 and 83.0 per cent of the embryos hatched (Fig. 3A). Exposed six hours earlier, only 36.0 per cent hatched ($P = 0.01$). This new condition followed only two weeks after the experiments that clearly showed a need for two pulses, and nine days after the embryos were placed in an artificial thermoperiod.

In an effort to locate the "time" of maximum sensitivity to photo-activation, each of 12 groups were exposed to a different two-hour period within the 24-hour thermocycle. After only four additional thermocycles the success of activation had declined from a maximum of 83.0 per cent to approximately one half that number (Fig. 3A). Two groups of embryos showed maximum sensitivity, one exposed at 0800, a time which corresponded with the thermal rise, and a second exposed eight hours later at 1600. Oddly, the total activation of embryos for the two periods of maximum sensitivity was equal to the number of embryos that had been activated at 1000 in the preceding experiment performed four days earlier.

Several interpretations are possible. The simplest seems to be that the embryos have retained only one phase of photosensitivity and that the rhythm of light sensitivity is responding to the thermocycle. One group of embryos seems to have retained a phase relation to the thermal rise, while a second group began

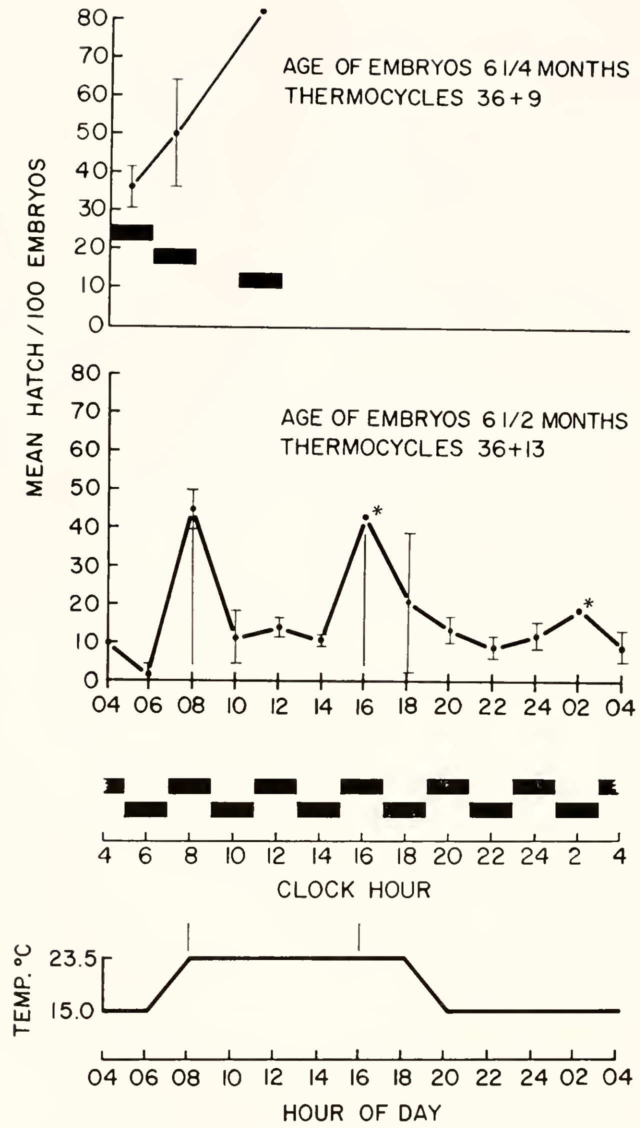

FIGURE 3. Hatching of embryos when given a single two-hour light pulse at various times in an artificial 24-hour thermal cycle. The embryos are from the same batch as shown in Figure 2 but are two weeks or more older. * S.E. is zero.

a synchronous drift to a later time, and at the time of measurement occupied no known relationship to the thermal cycle. The positions of the photo-sensitive phase were also unstable. After an additional month of incubation in the dark, the embryos were activated when exposed at nearly all phases of the thermocycle.

Disclosure of two distinct phases of light sensitivity will form the basis of later interpretation although the condition was unstable. Instability amounted to the loss of one of two inductive phases requiring light for activation. This was accompanied by the development of an asynchronous and seemingly continual sensitivity to light. The presence of two photo-inductive phases in each thermocycle was also unstable in a second series of experiments with a new batch of diapaused embryos.

In the second series the optimum interval between the two light exposures was clearly a function of when the embryos were first exposed to light pulses of two-hours duration. When exposed at the thermal rise, a 10-hour skeleton photoperiod was more effective than a 16-hour skeleton photoperiod. The reverse was true when the embryos were first exposed 12 hours after the thermal rise. These results are readily interpretable if the embryos become entrained to the thermal oscillations in the environment but do not distinguish the thermal rise (dawn) from the decline (sunset). It would seem that the first pulse of light was exposing the embryos at their "sunset" and the second pulse at "dawn." A delay in the first exposure to light would then result in an initial exposure of the embryos at dawn. In other words the interpretation suggests that the embryos may respond when "seeing" light first at either sunset or at dawn.

Substitution of potential synchronizers other than thermal oscillation was only partially successful. The simple transfer to fresh medium may have been effective. When exposed to a 2-hour pulse at the time of transfer, few (3.3 per cent) embryos were activated. When exposed 24 hours later, 70.0 per cent were activated. Exposure at both times (hours 0 to 2 and 24–26) was ineffective (9.3 per cent). Attempts to synchronize with light were disastrous. Single exposures of 15 minutes to embryos still in the original medium damaged the embryos. A small number (15.6 per cent) hatched but most of the affected (activated?) embryos disintegrated. Hourly exposures to the safelight (red light) at 24-hour intervals were only slightly successful.

The foregoing results demonstrated that light may be required for activation. They clearly show that the *Daphnia* embryo may be discontinuously sensitive to light. Specific times of light sensitivity appear to bear a specific relationship to a natural or imposed thermocycle. Two conditions of photo-sensitivity were revealed. In one condition following the onset of the photo-sensitive phase of diapause, the embryos may require exposure to light at two distinct times within one thermocycle. One of these times may be interpreted as the dawn phase of the embryo's putative daily cycle, the second as the sunset phase. In a second condition the embryo requires only a single exposure to light. Whether the single exposure serves as dawn or sunset cannot be interpreted, as a result of a seemingly ambivalent phase relationship of the embryo's endogenous cycle to the thermocycle of the environment.

Photo-sensitizing with elevated tensions of CO_2

Daphnia embryos held in darkness in a flowing stream (Fig. 1) for up to 14 months retain their viability but are photo-refractory. They may be made photo-sensitive with manipulation of the gaseous environment. Following incubation in a medium bubbled with nitrogen, photo-activation is possible but only when the tension of CO_2 is elevated by bubbling the medium with air enriched with 5.0 per cent CO_2; detailed results are given below. The gas modification is a well tested procedure for breaking the dormancy of many organisms including certain parasites (Fairbairn, 1961; Rogers and Sommerville, 1968) and seeds (Ballard, 1958, 1967; Ballard and Grant Lipp, 1969). Although some essential details are still lacking, the procedure may guarantee a uniform response from embryos incubated for six months to 14 months in the dark before exposure to light.

The function(s) of CO_2

Elevated CO_2 tensions may perform two functions in the parasite (Sommerville, 1964). One is to break the dormancy and the second is to initiate a development leading to the molt of the diapausing instar. Sommerville (1964, 1966) showed the second function could be largely completed within the first 24 hours of incubation. A fungal parasite of cucumbers needs both a light and a dark-requiring process in order to form spores (Barnett and Lilly, 1950, 1955). The dark-requiring process is suppressed by what has been identified as an elevated tension of CO_2 in the culture vessel.

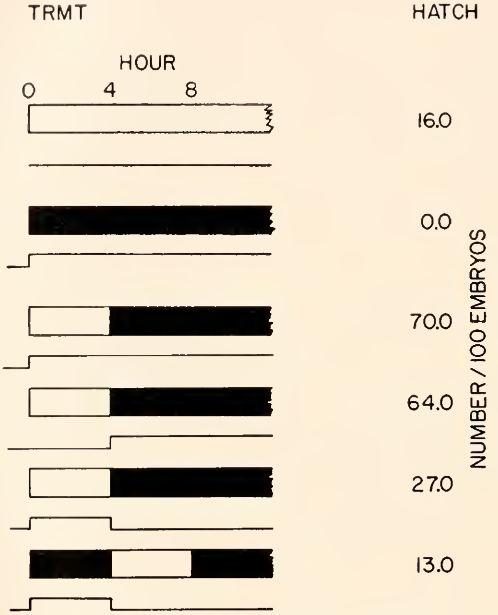

FIGURE 4. Photo-activation in the presence of an elevated CO_2 tension. The medium has been enriched with a mixture of 5.0 per cent CO_2 in air. Embryos were in constant dark and temperature for ten months, six of which were in the "flowing stream" and four months prior to treatment in a medium treated initially with 100 per cent N_2. Note some activation independent of CO_2 enrichment.

The two functions of CO_2 may not be separable in the activation of *Daphnia* embryos. It is clear that both light and elevated CO_2 tensions participate in the activation process. Moreover, the action(s) of CO_2 is effected in the dark. The exposure of embryos to a four-hour pulse of light either with or before elevation of CO_2 resulted in a high level of activation (Fig. 4). Conversely, pulsing the embryos with CO_2 for four hours either with or before the light pulse had little or no effect. In the experiment the level of hatching in the CO_2 control was significantly greater than zero (16.0 per cent). This background level was eliminated in subsequent experiments by restricting the duration of incubation in nitrogen. Whatever the function of CO_2 in the dark, the presence of CO_2 restricts the light requirement to a maximum of four hours.

A working hypothesis of activation is that the embryos may require stimulation at two separate inductive phases of a daily cycle as shown in earlier experiments (see above). One of the phases has an absolute requirement for light. The second phase might be inducible with light or a substitute such as an elevated tension of CO_2. Results of the first experiment with CO_2 showed that CO_2 renders the embryo photo-sensitive. That CO_2 later substituted for a second pulse of light is an assumption consistent with the dual role of CO_2 as shown by Sommerville (1964) and consistent with the observation that CO_2 suppresses a dark (short-day) reaction (Barnett and Lilly, 1955). Thus it may be proposed that CO_2 may have two roles in the activation of the *Daphnia* embryo: to break photo-refractoriness and to substitute for light at one of two photo-inducible phases.

Results supported the hypothesis that CO_2 substitutes for light at one of two inductive phases of a daily cycle, although in a way more striking than anticipated. Ten groups of embryos in triplicate were given an elevated level of CO_2 under safelight. The first group was exposed immediately to a two-hour pulse of light and the other nine were similarly exposed but at a later time in the following 32-hour interval (Fig. 5). Embryos were activated in all but the control groups and ranged from 44 to 100 per cent with no trend apparent. That is the embryos would appear to be photo-inductible at all times tested.

The striking features of hatching were the synchrony and the length of the interval from light exposure to the time of hatching (Fig. 5). The interval assumed two basic patterns. When the embryos were exposed to a light pulse at any of four times in the first 12 hours, they hatched simultaneously and 83 hours after CO_2 elevation, that is they behaved as though CO_2 was the stimulus triggering activation. Controls clearly showed that both light exposure and CO_2 elevation were necessary for activation. In the subsequent 12-hour interval, that is from hours 12 to 24 following CO_2 elevation, the pattern of hatching suggested that light now provided the key stimulus. The situation reversed itself in the third 12-hours and once again the pattern of hatching suggested CO_2 was the key stimulus.

The observed pattern of hatching strongly suggests that CO_2 triggers a dark reaction. It may be supposed that light given during this phase initiates no immediate effect but that the light stimulus is "stored" until the beginning of a second phase at which time a second necessary process is initiated by light following which the embryo begins an irreversible development. If the embryos are not exposed to light until the second 12-hour period, the process of activation that was initiated with CO_2 apparently stops and waits for the light to initiate the second (light requiring) process. Since photo-activation is here suggested to occur at dawn, the second 12-hour period might be equivalent to Bünning's photophase. Now the hatching response to light exposure in the interval from hours 24 to 34 is the exciting thing since it strongly suggests an endogenous rhythm of recycling of the embryos to a state comparable to when the CO_2

tension was first elevated. Again CO_2 seems to activate a dark reaction and the embryo stores the light signal for dawn use. It is to be noted that the overall interval from light exposure to hatching is now shortened by approximately eight hours, almost as if in the first 24 hours some development had taken place in the presence of CO_2 but in the absence of light.

One essential demand of this interpretation is that CO_2 initiates a dark reaction and that initiation is independent of the time at which the embryos are introduced to the CO_2 elevation. To test this the same type of experiment as the preceding was repeated with four sets of embryos, with successive sets receiving the CO_2 elevation at hour 0, 6, 12 and 24. All sets were removed from the same container, which came from a stagnant medium, since the source in the flowing stream had been exhausted.

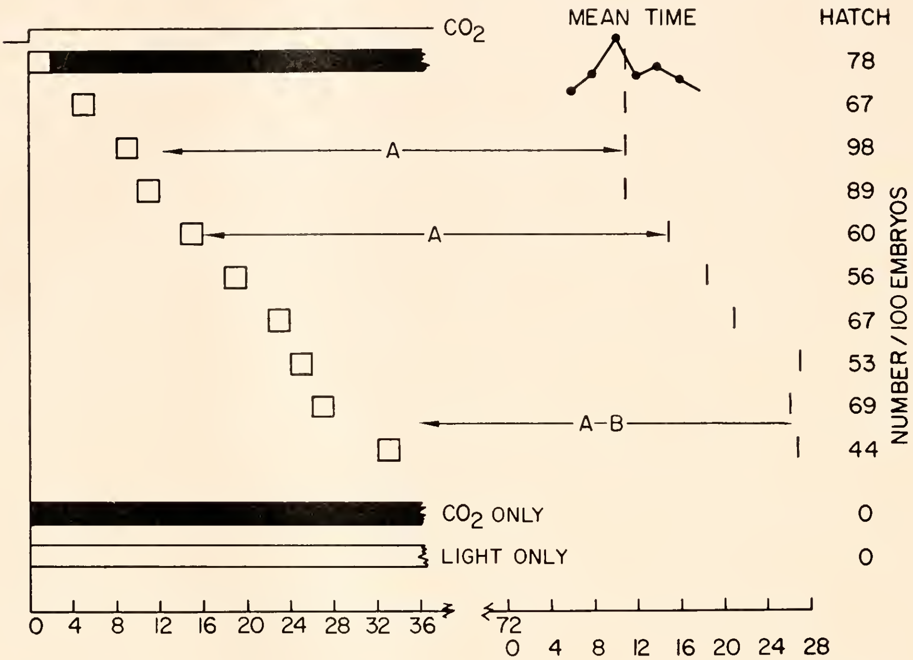

FIGURE 5. Mean hatching time of embryos exposed to a two-hour pulse of light at some time in a 34 hour interval following CO_2 elevation of the medium. Previously, embryos had spent three weeks in a N_2 environment following transfer from the "stream." "A" is the (variable) interval from onset of light to mean hatching time. "B" is the (fixed?) interval by which total development time (A) has been shortened in the second cycle following CO_2 elevation.

With one important exception the embryos responded as predicted. Following gasing the embryos began development which advanced them a maximum of 13 hours toward hatching and there appeared to be no qualitative difference in any of the four groups (Fig. 6). Unlike the results of the preceding experiment, the embryos continued to develop for the first 20 hours following gasing instead of for the first 12 hours. Also unlike the preceding experiment, all but the group started at hour 12 began a reversal at hour 20 following CO_2 elevation. At hour 32 the groups started at hours 0 and 6 had reversed development to the point where hatching required the same duration as though light and the CO_2 elevation had been given simultaneously. Following hour 32 the two groups again began development toward hatching. Only the group started at hour 12 behaved similarly to the embryos in the preceding experiment in that development executed during the first 20 hours was retained throughout the subsequent 16 hours. With significant quantitative exceptions the experiment is believed to support the hypothesis that CO_2 triggers one of two essential processes necessary for termination of the diapause.

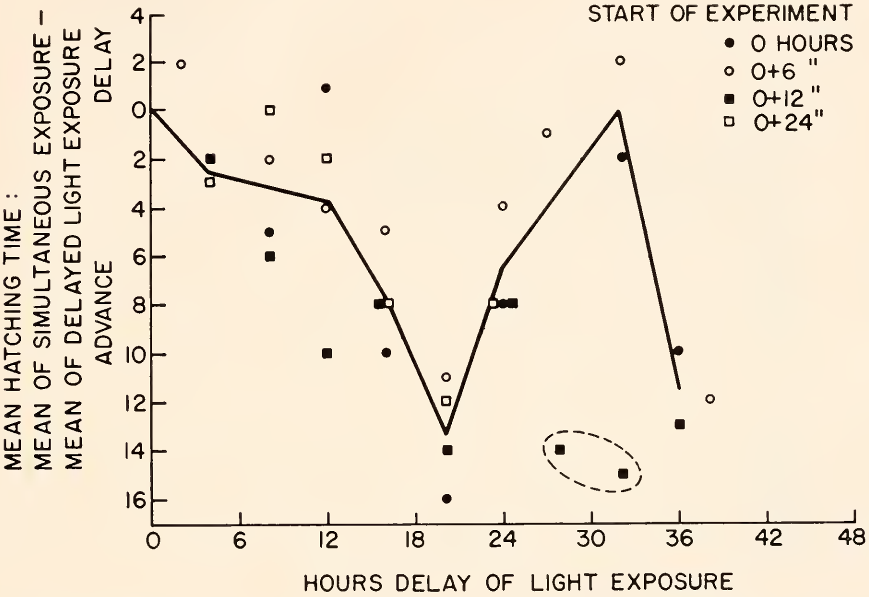

FIGURE 6. Mean hatching time of embryos given a two-hour light pulse simultaneous with CO_2 enrichment or at some time within 34 hours following enrichment. Four sets of embryos were treated with CO_2 at hour 0, or at 6, 12, or 24 hours later. Hatching time is expressed in hours from light exposure but relative to the hours required when both light and CO_2 treatments were given simultaneously. Embryos previously incubated for one year in a stagnant environment in constant dark.

The hypothesis also implies that the signal from the light pulse may be "stored" for initiation of the light requiring reaction following completion of the CO_2-initiated reaction. Experimental evidence is supportive. When CO_2 is withdrawn at hour 10 following the elevation, activation is not significantly greater than zero despite a light pulse given with the CO_2 elevation (Fig. 7). However, when withdrawal is followed immediately by a second light pulse (hour 10 to 12), activation is achieved and the embryos hatch. If four hours are allowed to elapse between withdrawal of CO_2 and exposure (hours 14 to 16) to light, no embryos are activated indicating that a CO_2 controlled process is reversible. These results strongly support the demand for induction of two temporally separate processes, one of which is satisfied by CO_2 and the second of which requires light. They also show the order in which the two inducible processes must be satisfied when CO_2 is employed to break the photo-refractory phase of diapause.

Breaking diapause with modification of the gaseous environment

When the embryos are incubated in a "stream," enrichment of CO_2 concentration becomes a necessity for photo-sensitivity as shown above. CO_2 treatment was not sufficient by itself, however. Although the details are still being investigated, the embryos require some preliminary exposure to a low O_2 environment. Following six months incubation in the "stream" a collection of embryos was divided into two groups. One group was subjected to a 100% N_2 environment for two weeks, the second to a 50% CO_2, 20% O_2 environment for the same interval. Both groups of embryos were then exposed to light with or without CO_2 elevation (5% CO_2 in air). The groups treated with N_2 gave an 87.0 per cent hatch when exposed to both light and elevated CO_2, the CO_2 control, 0.0 per cent. The groups treated with 50% CO_2 and normal O_2 gave no hatching in light and 5 per cent CO_2, although there was a 6.0 per cent hatch in the CO_2 controls. The low O_2 (N_2) environment may be considered a prerequisite to the effectiveness of CO_2 elevation in the presence of light.

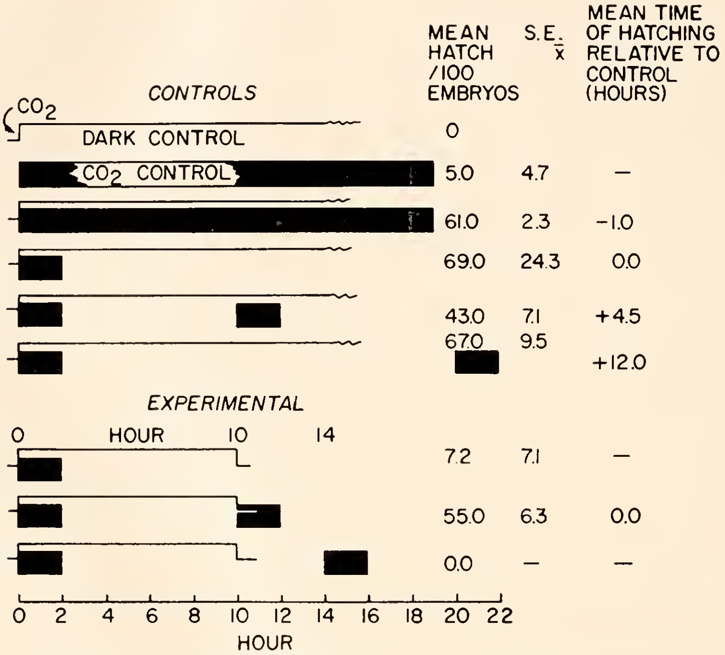
MEAN HATCH /100 EMBRYOS
S.E. x̄
MEAN TIME OF HATCHING RELATIVE TO CONTROL (HOURS)
CONTROLS
CO2
DARK CONTROL
0
CO2 CONTROL
5.0
4.7
—
61.0
2.3
-1.0
69.0
24.3
0.0
43.0
7.1
+4.5
67.0
9.5
+12.0
EXPERIMENTAL
0
HOUR
10
14
7.2
7.1
—
55.0
6.3
0.0
0.0
—
—
0 2 4 6 8 10 12 14 16 18 20 22
HOUR

FIGURE 7. "Storage" of the light stimulus by CO_2 as shown by the need for a second light exposure when CO_2 is withdrawn following ten hours after CO_2 elevation and the initial light exposure.

The low O_2 and high CO_2 environment, which occurs normally with aerobic metabolism in a closed system may therefore act independently in breaking the photo-refractory phase of diapause in *Daphnia.* Presumably this is what happens in a stagnant medium and ultimately results in the disappearance of one of the two inducible phases within the daily cycle of the embryo. Ballard and Grant Lipp (1969) have shown that a low O_2 atmosphere achieves partially the effect of an elevated CO_2 atmosphere in breaking the dormancy of clover seeds. A preliminary exposure to low O_2 was not necessary for CO_2 effectiveness in their material, however.

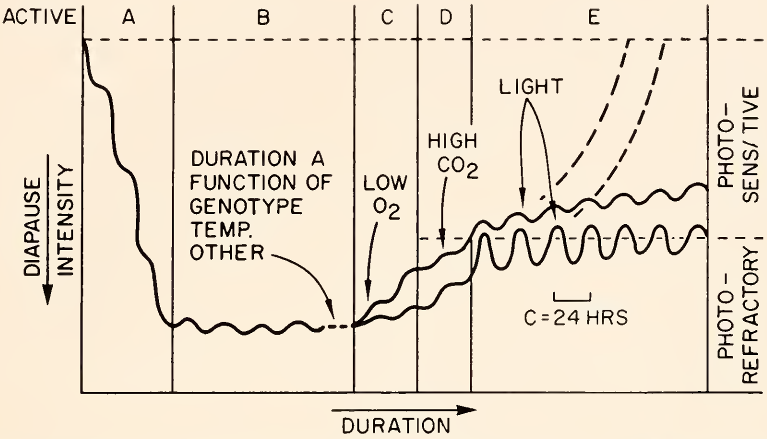

FIGURE 8. Schematic model of diapause interval in embryos of *Daphnia pulex.* Each of four states (A-D) are identified by the changing responsiveness of the embryos to specific stimuli in the environment, including temperature, O_2 and CO_2 tensions, and light. In a fifth state (E) the embryo is photosensitive. The light requirement for terminating the diapause may differ depending on the manner of entry to the photo-sensitive phase of diapause. (See text for details.)

A further complexity is that the *Daphnia* embryos are not immediately sensitive to the low O_2 stimulus. Some interval of incubation in the stream is a necessary preliminary condition for response to low O_2 after only two weeks. Four weeks of exposure was indaquate when the embryos were removed from the culture vessel and placed directly in low O_2. Preliminary experiments suggest that the embryos become primed for response to the low O_2 environment if the flow of medium in the stream is interrupted periodically.

Photo-refractoriness in the diapaused embryo may represent a variety of internal states, if responsiveness to the variety of stimuli employed in this and other studies may be used as criteria. A descriptive model (Fig. 8) of diapause suggests a minimum of five states, the first four (A-D) of which are photo-refractory. In the initial (A) state the diapause intensifies or deepens. During this state progressively fewer embryos are rendered photo-sensitive following such treatments as osmotic shock (Stross and Hill, 1968), decapsulation (Davison, 1969) *etc.* The following interval (B) is one of intense diapause, the duration of which may be determined by temperature, genotype of the embryos, and, in the case of the strain used in this study, some unknown set of conditions.

A third state (C) is marked by the responsiveness of the embryo to low O_2 tensions, the effect of which is to induce a state (D) of sensitivity to a stimulus such as elevated CO_2. The photo-refractory phase of diapause may be considered at an end when the "D" state has been satisfied. Since the light sensitizing action of CO_2 is reversible, the embryo may move reversibly from state C to state E, the final state, with the addition or withdrawal of CO_2.

In the final or E state the embryo is fully photosensitive and exposure of the embryo to light at the appropriate phase of an endogenous cycle will trigger activation. The final state may be forced with manipulation of the gaseous environment, and it is suggested that only some strains of *D. pulex* are amenable, or it may result from incubation at the appropriate temperature.

Alternative states of photo-sensitivity in the "E" phase of diapause development are described in Figure 8. In one state (bottom line) the embryo is photo-inducible for only a part of a daily cycle which may include two (*e.g.,* Fig. 2) or one (*e.g.,* Fig. 3) phase of induction. In the alternative state (top line) the embryo may be continually sensitive to light during its "E" phase of diapause. Although sensitivity may be continuous, the rhythmic pattern of hatching (Figs. 5 and 6) would suggest that photo-induction is also rhythmic, that is, the product of a photo-reaction is apparently "stored" until needed in an internally ordered activation process.

Discussion

The diapaused embryo of *Daphnia* is potentially under the control of photoperiod, as may be the entire life cycle. The requirement for light is readily demonstrable. However the requirement for one inductively long day may be obscured by the internal state of the embryo. In one state induction clearly requires one long day or two pulses of light that simulate one long day. The embryo may therefore have two, not one, photo-inducible phases in each endogenous daily cycle. Scant evidence suggests that either the dawn or sunset stimulus may be received first.

In a second state only one light pulse of two hours is sufficient for inducing a return to active embryonic development. In this state the embryo is still manifesting time measurement since it is activated by the light pulse only at a certain time(s) or phase of a 24-hour thermocycle. The loss of one of two photo-inducible phases marks a second internal state and follows a first. Conceivably some arthropods have normally only one photo-inducible phase and its "loss" permits diapause termination in constant dark. There are many such examples of termination in constant dark and they include another strain of *Daphnia pulex* (Stross and Hill, 1968) as well as other arthropods, aquatic (Paris and Jenner, 1959) and terrestrial (Williams and Adkisson, 1964). That two photo-inducible phases may exist in arthropods other than *Daphnia* is demonstrated by the response of the aphid *Megoura* to photoperiod (Lees, 1966).

Other recent studies infer photo-induction of diapause termination at more than a single phase of a daily cycle. Hamner (1969), employing a regimen of one short day followed by a cycle of complete darkness, shows that night interruptions in the first night period are more effective in creating a long-day than are light interruptions at the appropriate time in the second cycle. A light-influenced preparative process, the product of which decays following the end of the main light period, was inferred. Saunders (1970) using the same approach with a different insect described an opposite result in which interruptions during the second (and third) cycle were apparently more effective. Both studies suggest that two light controlled reactions may be involved in each inductive cycle.

The reaction to light at supposedly two phases, one at sunset and the other at dawn, is consistent with the response to night interruptions when given in 24-hour cycles. When the main light period is suitably short (6 to 10 hours) there are normally two intervals in the night when a light interruption is inductive. Exposure to light early in the night may be inductive when it forms the sunset of a long day. Exposures in the late night are effective when they form the dawn of a long day (Pittendrigh and Minis, 1964; Pittendrigh, 1966). Light exposure in the late-night is usually the more effective inducer of a long daylength (Adkisson, 1964, 1966; Saunders, 1968, 1970) presumably because the main light period now forms the sunset which may be interpreted as the phase of photo-inducibility. However, Pittendrigh (1966) strongly suggests the photo-inducible phase may be the late-night or dawn period, a view supported by the interpretation of the results in this paper. In that event the main light period would be involved with a second inductive phase which could be preparative for a second light reaction which may take place at dawn.

The existence of a second inducible phase that occurs near (sunset) the end of the light period and which provides an essential product for a photo-induction that occurs at dawn may be supported by temperature manipulation. Experiments that combine a period of chilling with night interruptions have shown that chilling during the day may interfere with the preparation while those given at night seem to preserve the product of the preparative process. At least such an interpretation may be deduced from the results as described by Saunders (1968) and Saunders and Sutton (1969).

The deliberate elevation of CO_2 tension, which may be necessary to break photo-refractoriness, introduces an additional complexity. The embryo now responds to a light stimulus as though it were continuously photo-inducible. However, the pattern of hatching suggests that the embryo may be photo-inducible only at certain phases of some internal cycle. To explain the continued sensitivity to light, it is suggested that some intermediate product is stored for a phase-specific process of activation. Storage apparently requires the presence of the elevated tension of CO_2.

Three potential functions of CO_2 in terminating the diapause of *Daphnia* are described in an extension of the so-called coincidence model (Pittendrigh and Minis, 1964) (Fig. 9). One function is to break the photo-refractory state of the embryo. The second is to store a light signal. The third function is to preserve for all, or a part, of each daily cycle a product of some dark reaction until the embryo is exposed to light. The three (x, y, z) functions of CO_2 are deduced from the light requirements of the embryo in state one when there may be two photo-inductive phases each cycle.

The coincidence model describes photo-induction as a light activated enzyme converting a substrate when the concentration of the substrate achieves threshold concentration and that occurs once each circadian cycle. An extension (Fig. 9) describes two such substrate conversions, the products from each combining to form a final product. One intermediate product (P_{I_1}) reacts with a second intermediate (P_{I_2}) to form the final product (P_F), provided the concentration of P_{I_2} is sufficient, as would be the case if the night were short. Viewed in these terms, the potential functions of CO_2 are easily described.

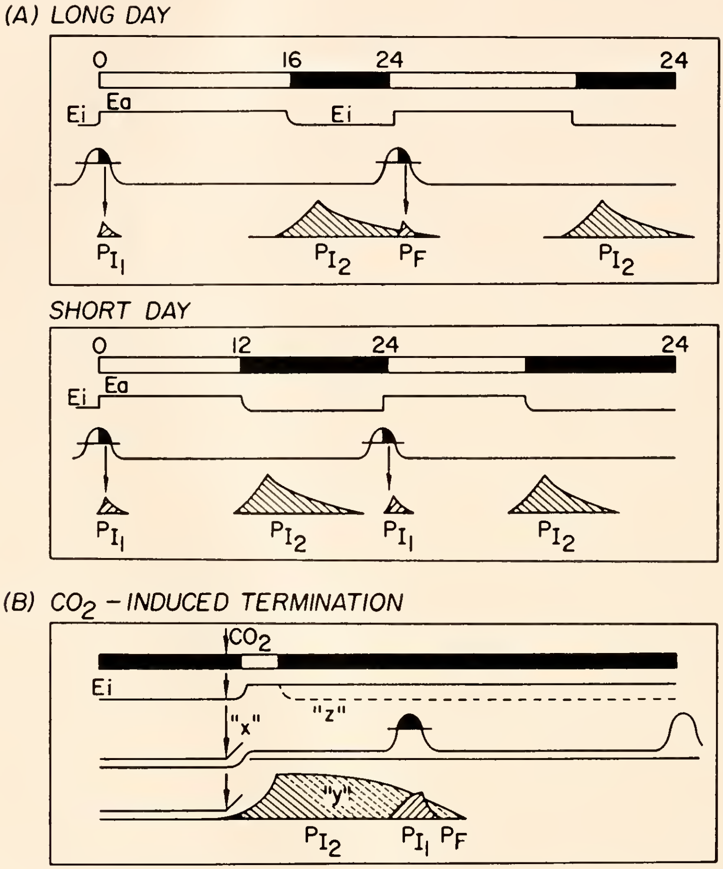

FIGURE 9. A model of photoperiodism describing the potential functions of CO_2. The basic model is an extension of the coincidence model to include two "photo-inducible" phases in each daily cycle. Three functions of CO_2 are postulated to explain experimental observations. They are the breaking of photo-refractoriness ("x"), substitution of one light stimulus ("y"), and "storage" of a light signal until the embryo becomes photo-inducible ("z"). See text for details.

The first function of CO_2 "unlocks" the machinery for making two intermediate products, shown in Figure 9B as the "x" function. This effect is analagous to excystment in parasites (Sommerville, 1964, 1966). The second function, "y," is to preserve the product of the dark reaction which provides the second substrate (P_{I_2}). This effect of CO_2 could be analagous to the preventative effect of CO_2 in short-day (long-night) induction (Barnett and Lilly, 1950, 1955) and possibly to the second function of stimulating molting in certain parasites. The third function, "z," is the presumed storage of the light signal until an essential substrate reaches critical concentration.

Whatever the functions of CO_2, it seems clear that termination of diapause in the *Daphnia* embryo may be controlled by daylength. Termination of the entire population in nature may therefore be synchronous. Synchrony is more likely to occur when the gaseous environment of the embryos is appropriately close to equilibrium with the atmosphere.

Mr. Timothy Downing provided technical assistance. Dr. C. Edwards and Dr. J. Jacklet reviewed the manuscript and Dr. Jacket was instrumental in formulating the model of photoperiodism. Dr. J. Mackiewicz provided references to the literature on parasites. The author is grateful to these and others not specificially mentioned. Research supported in part from grants of the NSF and Joint Awards Council, State University of New York.

SUMMARY

The diapaused embryo of the cladoceran, *Daphnia pulex,* may require light for terminating the diapause, and a single photocycle may be adequate. In *D. pulex* the light-refractory phase of diapause may be broken or completed in a variety of environments. In the Supply House strain employed in these studies, the usual low temperature treatment is unnecessary when the embryos are placed in constant darkness in sealed containers. Alternatively, the refractory state in constant darkness may be broken with low O_2 and high CO_2 tensions. Both modifications were shown to be necessary and in that sequence.

A single long-day light signal may terminate the diapause when the embryo passes from the photo-refractory to the photo-sensitive phase. Three kinds of light responses were observed, however. Each relates to the treatment given the embryos prior to light exposure.

In one so called internal state the embryo requires one long day or two pulses of light. Scant evidence suggests that the first pulse may be interpreted as either dawn or sunset. In this state there are obviously two photo-inductive phases in each inductive cycle. The embryo may change, however, by "losing" one of the photo-inductive phases. In this condition a single two-hour pulse of light, if given at the appropriate time of a thermocycle, may terminate the diapause.

A third internal state is introduced when CO_2 is employed to break the refractory phase of diapause. The diapause is terminated by a single two-hour pulse of light, and the embryo is continuously sensitive to the light stimulus. However, the pattern of hatching indicates that activation may occur at restricted phases within the induction cycle.

Several roles of CO_2 may be deduced from the experimental results. An elevated CO_2 tension breaks photo-refractoriness. It also induces a "dark"

reaction which may need to be completed before the light may stimulate termination of the diapause. Withdrawal of CO_2 after 10 hours prevents activation unless a second light pulse is given immediately thereafter. The apparent deferral of light stimulation suggests a third function of CO_2, that of "storing" the light signal until the embryo becomes photo-inductive.

The potential roles of CO_2 are described in a model of photoperiodism that requires photo-induction at two phases in each daily cycle.

LITERATURE CITED

Adkisson, P. L., 1964. Action of the photoperiod in controlling insect diapause. *Amer. Natur.*, **98**: 357–374.

Adkisson, P. L., 1965. Light-dark reactions involved in insect diapause. Pages 344–350 in J. Aschoff, Ed., *Circadian Clocks.* North Holland Publ. Co. Amsterdam.

Adkisson, P. L., 1966. Internal clocks and insect diapause. *Science*, **154**: 234–241.

Ballard, L. A. T., 1958. Studies of dormancy in the seeds of subterranean clover (*Trifolium subterranean* L.) I. Breaking of dormancy by carbon dioxide and by activated carbon. *Aust. J. Biol. Sci.*, **11**: 246–260.

Ballard, L. A. T., 1967. Effect of carbon dioxide on the germination of leguminous seeds. Pages 209–219 in N. Borliss, Ed., *Physiology, Ecology and Biochemistry of Germination.* Ernst-Moritz-Arndt-Universität Press, Greifswald, East Germany.

Ballard, L. A. T., and A. E. Grant Lipp, 1969. Studies of dormancy in the seeds of subterranean clover (*Trifolium subterranean* L.) III. Dormancy breaking by low concentrations of oxygen. *Aust. J. Biol. Sci.*, **22**: 279–288.

Barnett, H. L., and V. G. Lilly, 1950. Nutritional and environmental factors influencing asexual sporulation of *Choanephora cucurbitarum* in culture. *Phytopathology*, **40**: 80–89.

Barnett, H. L., and V. G. Lilly, 1955. The effects of humidity, temperature and carbon dioxide on the sporulation of *Choanephora cucurbitarum. Mycologia*, **47**: 26–29.

Bunning, E., 1959. Physiological mechanism and biological importance of the endogenous diurnal periodicity in plants and animals. Pages 507–530 in R. B. Withrow, Ed., *Photoperiodism and Related Phenomena in Plants and Animals.* American Association for the Advancement of Science Symposium #55, Washington, D. C.

Bunning, E., 1964. *The Physiological Clock.* Academic Press, New York, 145 pp.

deWilde, J., C. S. Duintjer and L. Mook, 1959. Physiology of diapause in the adult Colorado beetle. I. The photoperiod as a controlling factor. *J. Insect Physiol.*, **3**: 75–85.

Davison, J., 1969. Activation of the ephippial egg of *Daphnia pulex*. *J. Gen. Physiol.*, **53**: 562–575.

Fairbairn, D., 1961. The in vitro hatching of *Ascaris lumbricoides* eggs. *Can. J. Zool.*, **39**: 153–162.

Hamner, W. M., 1969. Hour-glass dusk and rhythmic dawn timers control diapause in the codling moth. *J. Insect Physiol.*, **15**: 1499–1504.

Lees, A. D., 1966. Photoperiodic timing mechanisms in insects. *Nature*, **210**: 986–989.

Minis, D. H., and C. S. Pittendrigh, 1968. Circadian oscillation controlling hatching: Its ontogeny during embryogenesis of a moth. *Science*, **159**: 534–536.

Paris, O. H., and C. E. Jenner, 1959. Photoperiodic control of diapause in the pitcher-plant midge. *Metriocnemus knabi.* Pages 601–624 in R. B. Withrow, Ed., *Photoperiodism and Related Phenomena in Plants and Animals.* American Association for the Advancement of Science Symposium #55, Washington, D. C.

Pittendrigh, C. S., 1966. The circadian oscillation in *Drosophila pseudoobscura* pupae: a model for the photoperiodic clock. *Z. Pflanzenphysiol.*, **54**: 275–307.

Pittendrigh, C. S., and D. H. Minis, 1964. The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *Amer. Natur.*, **98**: 261–294.

Rogers, W. P., and R. I. Sommerville, 1968. The infectious process and its reaction to the development of early parasitic stages of nematodes. Pages 327–348 in B. Dawes, Ed., *Advances in Parasitology.* Academic Press, New York.

SOMMERVILLE, R. I., 1964. The effect of carbon dioxide on the development of third stage larvae of *Haemonchus contortus in vitro*. *Nature*, **202**: 316–317.

SOMMERVILLE, R. I., 1966. The development of *Haemonchus contortus* to the fourth stage *in vitro*. *J. Parasitol.*, **52**: 127–136.

SAUNDERS, D. S., 1968. Photoperiodism and time measurement in the parasitic wasp, *Nasonia vitripennis*. *J. Insect Physiol.*, **14**: 433–450.

SAUNDERS, D. S., 1970. Circadian clock in insect photoperiodism. *Science*, **168**: 601–603.

SAUNDERS, D. S., AND D. SUTTON, 1969. Circadian rhythms in the insect photoperiodic clock. *Nature*, **221**: 559–561.

STROSS, R. G., 1965. Termination of summer and winter diapause in *Daphnia*. *Amer. Zool.*, **5**: 701.

STROSS, R. G., 1966. Light and temperature requirements for diapause development and release in *Daphnia*. *Ecology*, **47**: 368–374.

STROSS, R. G., 1969. Photoperiod control of diapause in *Daphnia*. III. Two-stimulus control of long-day, short-day induction. *Biol. Bull.*, **137**: 359–374.

STROSS, R. G., AND J. C. HILL, 1968. Photoperiod control of winter diapause in the freshwater crustacean, *Daphnia*. *Biol. Bull.*, **134**: 176–198.

WELLSO, S. G., AND P. L. ADKISSON, 1964. Photoperiod and moisture as factors involved in the termination of diapause in the pink bollworm, *Pectinophora gossypiella*. *Ann. Ent. Soc. Amer.*, **57**: 170–173.

WILLIAMS, C., AND P. L. ADKISSON, 1964. Physiology of insect diapause. XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkworm *Antheraea pernyi*. *Biol. Bull.*, **127**: 511–525.

Reference: *Biol. Bull.*, **140**: 156–165. (February, 1971)

OXYGEN POISONING IN THE ANNELID *TUBIFEX TUBIFEX*. II. OSMOTIC PROTECTION[1]

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Walker (1970) studied the effects of hyperbaric oxygen on *Tubifex tubifex*, an Annelid normally living in environments with extremely low oxygen tensions. These animals were killed by exposure to four atmospheres absolute oxygen pressure for 15 hours but recovered from exposures of up to eight hours or when longer exposures were interrupted by a sufficient interval at atmospheric conditions (Walker, 1970). In the present study the ability of various agents to modify the toxic effects of oxygen on *T. tubifex* has been investigated.

MATERIALS AND METHODS

T. tubifex was exposed to four atmospheres absolute oxygen pressure by the procedures described previously (Walker, 1970). The water used in this study both for worm exposures and for preparation of reagents was tap water which had passed through activated charcoal filters and to which 0.05 g disodium dihydrogen ethylenediamine tetraacetic acid (versene, Hach Chemical Co.) per liter was then added.

The effects of various agents were determined by adding 0.5 ml of the test solution to each depression of the paraffin block which already contained 4.5 ml water and one worm. Addition of agents was made immediately before oxygen exposure except where otherwise noted. In some experiments the effect of complete removal of the environmental water after oxygen exposure and replacement with another medium was tested. When heat stress experiments were run worms were exposed to increased temperatures in a water bath but were returned to room temperature to score survival.

Worm responses were judged by the following criteria: the presence or absence of movement and the presence of damage visible without a microscope. Worms having progressive damage and showing no movement for several observation periods were scored as dead. Such worms either became a grey, opaque, motionless mass or disintegrated completely. An asterisk following data for per cent survival in the tables indicates that the value does not correspond to an actual observation time. Such data were obtained from a graph of the per cent survival versus time after oxygen exposure for the experiment in question.

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[1] This paper is a portion of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Illinois, Urbana, Illinois.

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RESULTS

1. *Protection by solutes*

The possibility that glycolysis might be inhibited by hyperbaric oxygen in *T. tubifex* was first considered. Worms in either one per cent glucose or in water were exposed to oxygen in two doses interrupted by various intervals. These preliminary glucose experiments were a portion of the dosage fractionation experiments reported previously (Walker, 1970). In one experiment glucose was added during the interval following the first oxygen exposure. In another experiment worms were exposed to oxygen in water, in one per cent glucose, in 2,4-dinitrophenol at a final concentration of $1 \times 10^{-4}\,M$ and in one per cent glucose plus $1 \times 10^{-4}\,M$ 2,4-dinitrophenol. The results of these experiments are presented in Table I.

TABLE 1

Survival of T. tubifex *as affected by addition of glucose and of dinitrophenol during fractionated oxygen exposures (16 worms per group)*

Worm group	Oxygen treatment			Post-exposure survival (per cent)	
	First dose hours	Interruption hours	Second dose hours	48 hours	153 ± 5 hours
Water	8	2	8	24.1*	0.0
1% Glucose (added at start of first exposure)				100.0	100.0
1% Glucose (added at end of first exposure)				51.7*	12.5
Water	10	4	8	12.5	9.0*
1% Glucose (added at start of first exposure)				91.1*	87.5
Water	8	6	8	75.0	68.8
1% Glucose (added at start of first exposure)				100.0	93.8
DNP $1 \times 10^{-4}\,M$ (added at start of first exposure)				97.7*	93.8
1% Glucose + DNP $1 \times 10^{-4}\,M$ (added at start of first exposure)				93.8	93.8

* By interpolation.

One per cent glucose had a striking protective effect on oxygen-treated *T. tubifex*. Glucose had some protective effect even when it was added after the first eight-hour oxygen exposure. Control experiments showed that 2,4-dinitrophenol at $1 \times 10^{-4}\,M$ was not lethal for unoxygenated *T. tubifex;* this concentration, reported to uncouple oxidative phosphorylation (Loomis and Lipmann, 1948), not only did not reverse the protective effect of glucose but appeared to have some protective effect of its own.

T. tubifex were also exposed to oxygen in one per cent Proteose-Peptone (Difco) during the dosage fractionation experiments. This medium is very favorable for the growth of microörganisms. Worm depressions containing Proteose-Peptone rapidly became contaminated and the worms had to be discarded. Proteose-Peptone nevertheless provided a protection comparable to that of glucose during the period before fouling.

The protective effect of both glucose and Proteose-Peptone was immediately apparent from the behavior of oxygen-exposed worms when they were removed from the oxygen chamber. Worms exposed to oxygen in glucose or Proteose-Peptone were moving normally and were not contracted, while worms exposed in water were highly coiled and almost motionless.

Since such different substances as glucose, Proteose-Peptone and 2,4-dinitrophenol all protected *T. tubifex* against the toxic effects of oxygen and particularly since a moderate NaCl concentration was produced in dissolving and adjusting the pH of the 2,4-dinitrophenol solution, the possible protective effect of NaCl was suggested. Three groups of worms were therefore exposed to oxygen for 16 hours in water, in one per cent glucose and in NaCl at a concentration osmotically equivalent to the one per cent glucose and their post-exposure survival scored. Survival of *T. tubifex* at 219 hours after oxygen treatment was 25 per cent for worms exposed to oxygen in water, 100 per cent for worms exposed to oxygen in one per cent glucose and 87.5 per cent for worms exposed to oxygen in NaCl osmotically equivalent to the glucose. Again the protective effect was clear from the worms' behavior on removal from the oxygen chamber. Worms exposed to oxygen in either glucose or NaCl were more active and appeared more like normal worms than did the worms exposed to oxygen in water.

To determine the concentration providing maximum protection, worms were exposed to an 18-hour oxygen dose in solutions of glucose or NaCl ranging in concentration from 0.1 to 0.0001 *M*. Results of these experiments are presented in Table II.

Table II

Survival of T. tubifex *following 18 hours of oxygen exposure in various concentrations of glucose or sodium chloride (16 worms per group)*

Worm group	Post-exposure survival (per cent)			
	25 ± 4 hours	53 ± 1 hours	94 ± 5 hours	144 ± 3 hours
Water	62.5	62.5	43.8	37.5
Glucose 0.1 *M*	87.5	87.5	87.5	75.0
Glucose 0.01 *M*	87.5	87.5	87.5	81.3
Glucose 0.001 *M*	62.5	50.0	43.8	31.3
Glucose 0.0001 *M*	56.3	56.3	37.5	18.8
Water	8.3	8.3	8.3	8.3
NaCl 0.1 *M*	100.0	31.3	31.3	31.3
NaCl 0.01 *M*	100.0	87.5	87.5	81.3
NaCl 0.001 *M*	18.8	18.8	18.8	18.8
NaCl 0.0001 *M*	46.6	46.6	46.6	46.6
Water	25.0	18.8	18.8	18.8
NaCl 0.05 *M*	100.0	100.0	100.0	100.0
NaCl 0.02 *M*	100.0	100.0	100.0	100.0
NaCl 0.005 *M*	68.8	55.5*	43.8	43.8

* By interpolation.

It is clear that maximum protection resulted from treatment with glucose solutions between 0.1 *M* and 0.01 *M*. The NaCl solution giving the greatest protection had a concentration of 0.01 *M*. Sodium chloride at 0.1 *M* offered equivalent protection during the first day after exposure, but its beneficial effect decreased markedly during the following day. It is of interest that this relatively high concentration of NaCl was beneficial even though one half of the control worms not exposed to hyperbaric oxygen died in this concentration of NaCl within two days. When the NaCl optimum was more sharply defined, optimum and equivalent protection occurred in 0.05 *M* and 0.02 *M* solutions. These results are presented graphically in Figure 1. It is interesting to note that one per cent glucose used in all other glucose experiments has a molarity of 0.056; NaCl which is osmotically equivalent to one per cent glucose has a molarity of 0.028.

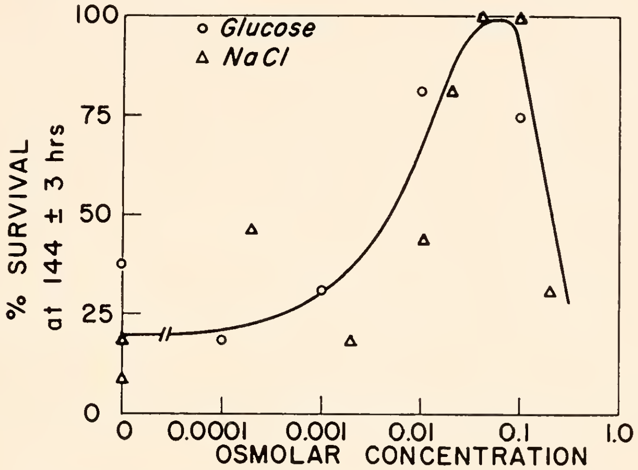

FIGURE 1. Survival of *T. tubifex* following 18 hours of oxygen exposure in various osmolar concentrations of glucose or sodium chloride.

Because of the marked protective effect of NaCl addition, it seemed essential to determine whether salts other than NaCl provided similar protection. Sodium nitrate and the chlorides of various monovalent and divalent cations were tested. All solutions were used at concentrations both above and below the osmotic equivalent of one per cent glucose. Complete dissociation of the salts at these concentrations was assumed. Results of these experiments are given in Table III.

These experiments indicate that protection against oxygen damage may be nonspecific in that salts with qualitatively different ionic composition had similar protective capacities. Calcium chloride at both concentrations and $MnCl_2$ at the lower concentration tested provided protection against hyperbaric oxygen equivalent to that produced by NaCl. Both concentrations of KCl and the higher concentrations of $CoCl_2$ and $MnCl_2$ were extremely toxic to the unoxygenated control worms. Most of the unoxygenated worms in the higher concentrations of KCl and $MnCl_2$ survived for two days but all were dead in five days. In 0.02 *M* KCl all the unoxygenated worms survived for six days but all were dead

TABLE III

Survival of T. tubifex *following 18 hours of oxygen exposure in various salt solutions (16 worms per group)*

Worm group	Post-exposure survival (per cent)					
	5 ± 1 hours	18 ± 1 hours	27 ± 3 hours	42 ± 2 hours	137 hours	185 ± 1 hours
Water	81.3	62.5	62.5	62.5	62.5	62.5
NaCl 0.05 *M*	100.0	100.0	100.0	100.0	93.4	93.4
NaCl 0.02 *M*	100.0	100.0	100.0	100.0	87.5	87.5
KCl 0.05 *M*	93.8	87.5	87.5	43.8	0.0	0.0
KCl 0.02 *M*	100.0	100.0	93.8	93.8	25.0	0.0
$NaNO_3$ 0.05 *M*	100.0	100.0	87.5	81.3	68.8	56.3
$NaNO_3$ 0.02 *M*	93.8	93.8	93.8	87.5	68.8	68.8
$CaCl_2$ 0.02 *M*	100.0	100.0	100.0	100.0	100.0	100.0
$CaCl_2$ 0.002 *M*	100.0	100.0	100.0	100.0	93.8	93.8
Water	100.0	25.0	25.0	18.8	18.8	18.8
$CoCl_2$ 0.02 *M*	100.0	62.5	0.0	0.0	0.0	0.0
$CoCl_2$ 0.002 *M*	100.0	43.8	6.3	6.3	6.3	6.3
$MnCl_2$ 0.02 *M*	100.0	75.0	0.0	0.0	0.0	0.0
$MnCl_2$ 0.002 M	100.0	87.5	81.3	81.3	81.3	81.3

at nine days; in 0.02 *M* $CoCl_2$ half the unoxygenated worms were dead in six days. All of the unoxygenated control worms survived in all other salt solutions. It is all the more impressive, therefore, that even those salts which were evenutally toxic to unoxygenated control worms delayed the death of oxygen-exposed worms during the first day following oxygen exposure. Sodium nitrate solutions produced a somewhat different effect. Protection by this salt was at first equivalent

TABLE IV

Survival of T. tubifex *as affected by replacement of the medium following 18 hours of oxygen exposure (16 worms per group)*

Worm group	Post-exposure survival (per cent)		
	16 hours	48 hours	250 ± 12 hours
Water not replaced	0.0	0.0	0.0
Water replaced with water	25.0	25.0	25.0
Water replaced with 0.028 *M* NaCl	81.3	86.8*	62.5
0.028 *M* NaCl not replaced	100.0	100.0	100.0
0.028 *M* NaCl replaced with 0.028 *M* NaCl	100.0	100.0	100.0
0.028 *M* NaCl replaced with water	93.8	93.5*	81.3
Water not replaced	12.5	12.5	0.0
Water replaced with water	12.5	8.5*	6.3
Water replaced with 1% Glucose	37.5	33.6*	18.8
1% Glucose not replaced	100.0	96.0*	87.5
1% Glucose replaced with 1% Glucose	100.0	100.0	81.3
1% Glucose replaced with water	100.0	100.0	43.8

* By interpolation.

to the protection given by NaCl. This protective effect gradually decreased until at the termination of the experiment the $NaNO_3$-treated worms had a mortality equivalent to that of the controls.

Results in Tables II, III and V indicate that some worms in water survived after uninterrupted oxygen exposures of 16 to 18 hours. A variation in sensitivity of *T. tubifex* to oxygen was observed throughout these experiments which could not be strictly correlated with increasing storage time in the laboratory but may be related to the adaptation previously reported (Walker, 1970).

2. *Time factor in protection*

Exchange experiments were set up in an attempt to determine when osmotic protection from NaCl or glucose addition was most effective. Worms were given an 18-hour oxygen exposure in water which was withdrawn in a syringe immediately after oxygen exposure and replaced with 0.028 *M* NaCl. Other worms were exposed to oxygen in 0.028 *M* NaCl which was replaced with water after oxygen exposure. A similar experiment was run using one per cent glucose. Results of these experiments are shown in Table IV.

TABLE V

Survival of T. tubifex *following addition of glucose or of sodium chloride at various times after 18 hours of oxygen exposure (16 worms per group)*

Worm group	Post-exposure survival (per cent)		
	12 hours	77 hours	205 hours
Water addition 0 time	60.0	53.4	33.7
1% Glucose addition 0 time	87.5	62.5	50.0
0.028 *M* NaCl addition 0 time	73.4	73.4	66.7
0.028 *M* NaCl addition ½ hour	100.0	86.7	86.7
0.028 *M* NaCl addition 1 hour	100.0	86.7	60.0
0.028 *M* NaCl addition 2 hours	80.0	73.4	66.7
0.028 *M* NaCl addition 4 hours	87.5	75.0	68.8
0.028 *M* NaCl addition 8 hours	68.8	62.5	62.5
0.028 *M* NaCl addition 12 hours	68.8	68.8	68.8

The presence of either saline or glucose during oxygen exposure resulted in a markedly increased worm survival. The continued presence of saline after removal from oxygen provided complete protection whether or not the saline was replaced by fresh saline solution. Even the replacement of saline with water after oxygen exposure did not greatly decrease worm survival. The presence of glucose after oxygen treatment greatly increased worm survival. Post-exposure replacement of glucose with water resulted in survival intermediate between the survival of worms treated continuously with water and worms in the continued presence of glucose. Addition of glucose after oxygen treatment may have a slight beneficial effect whereas addition of saline after oxygen exposure leads to markedly increased worm survival. Some worms which appeared to be moribund after exposure to oxygen in water resumed activity several hours after saline addition. Oxygen damage, however, was too great to be reversed completely and they eventually died.

The protective effect of post-exposure glucose and saline addition was substantiated by additional experiments; the data are presented in Table V.

Since addition of saline immediately following oxygen exposure resulted in a marked increase in worm survival, the next experiment was designed to test whether saline addition later in the recovery period would provide any protection and to determine at what time during the recovery period the saline addition would become ineffective. One trial was also run to determine whether treatment of the worms with saline (0.05 *M*) during the two hours previous to oxygen exposure in water had any beneficial effect.

Worms in 4.5 ml water were exposed to oxygen for 18 hours. A 0.5 ml volume of water or saline or glucose was added to each worm depression at the time of removal from oxygen or, in the case of saline, at various time intervals up to 12 hours after oxygen exposure. The final glucose concentration was one per cent; the final saline concentration was osmotically equivalent to that of the glucose.

The results, shown in Table V, indicate that saline addition as late as 12 hours after oxygen exposure enhanced survival. Glucose addition immediately after oxygen exposure resulted in survival intermediate between that of the controls exposed in water and that of worms treated with saline.

In another experiment a two-hour saline pretreatment had no protective effect; survival was indistinguishable from that of worms receiving no saline pretreatment.

3. Protection against other types of stress

The foregoing experiments showed that there is little specificity regarding the ions involved in protection against oxygen poisoning. It appeared equally important to determine whether protection is specific for oxygen poisoning or whether increasing the osmotic concentration of the environment would reduce mortality following other types of stress as well. To test this possibility, heat and hydrogen peroxide were used as stressing agents.

(a.) *Heat stress.* In preliminary tests worms heated for five minutes in a water bath at 45° C exhibited, within a few minutes, a typical pattern of response: marked hyperactivity followed by segmental constriction and segmental rupture which soon ended in worm disintegration. A five minute exposure at 38° C had no detectable effect other than to stimulate worm activity. Worms heated at 37° C for 60 to 75 minutes exhibited the constriction-rupture-disintegration response sequence spread over several days. Thus, prolonged exposure to 37° C offered more accurate visualization of the stress effects and appraisal of possible protective capacities of saline addition. In subsequent experiments, each worm was placed in either 5 ml water or 5 ml 0.05 *M* NaCl in a test tube. The tubes were heated in a water bath at 37° C for 75 minutes and subsequent responses of the worms were observed.

During the first day following the heat stress, worms in water without added salt exhibited a high mortality. Only 14.4 per cent of the heat-treated worms survived after 21 hours; none were alive at 103 hours. This response was not unlike the typical response following a fairly high dose of oxygen. Saline treatment resulted in a marked initial protection; 92.9 per cent of the worms exposed to heat stress in a saline environment were alive after 21 hours. Most of the saline-protected worms eventually died, however, so it appears that heat stress is not strictly comparable to oxygen damage at these dosages.

(b). *Hydrogen peroxide stress.* Hydrogen peroxide exposure was used as another type of stress. Preliminary experiments indicated that 20 μg per ml was a suitable hydrogen peroxide dose. Twenty four worms were therefore treated with 20 μg per ml hydrogen peroxide in combination with 0.05 M NaCl and their responses were compared to those of worms in hydrogen peroxide alone. After 640 hours 29 per cent of the worms in H_2O_2 and 78.3 per cent of the worms in H_2O_2 in combination with NaCl were alive. These data suggest that saline addition in combination with hydrogen peroxide has a beneficial effect on worm survival.

Discussion

It is difficult to account for the protective capacities of such a widely diversified collection of compounds as glucose, Proteose-Peptone, 2,4-dinitrophenol, NaCl, $CaCl_2$, KCl, $MnCl_2$, $CoCl_2$ and $NaNO_3$. Various ions, particularly divalent cations, have previously been reported to have a protective effect in oxygen poisoning. Magnesium *in vitro* (Dickens, 1946a) and *in vivo* (Wittner, 1957), manganese *in vitro* (Dickens, 1946a; 1946b) and *in vivo* (Wittner, 1957; Gerschman, Gilbert and Frost, 1958b) and cobalt *in vitro* (Dickens, 1946a; Horn, Williams, Haugaard and Haugaard, 1967) and *in vivo* (Wittner, 1957; Gerschman, Gilbert and Caccamise, 1958a; Gerschman *et al.*, 1958b) all were found to decrease the toxic effects of oxygen.

Several reports of protection by salts against other agents similar to the protection found in the present study have appeared in the literature. Freese, Bautz-Freese and Bautz (1961) found that when increasing concentrations of NaCl were present inactivation of phage T_4 by the mutagenic agent hydroxylamine was inhibited. Saier and Giese (1967) reported that the changes occurring in *Paramecium multimicro-nucleatum* caused by exposure to ultraviolet irradiation were inhibited or delayed as the salt content of the medium was increased. Some protection was obtained when glucose solutions osmotically equivalent to the salt solutions were tested. The medium providing the best protection was hypotonic to the organism but high in calcium and magnesium.

T. tubifex is a freshwater dweller; the tap water used in the present study containing the disodium salt of versene at an effective osmotic concentration of 4.5×10^{-4} M without other added solutes therefore corresponds closely to the worms' natural environment. Palmer (1968) reported that 10 per cent sea water (equivalent to 0.058 M NaCl; Prosser, 1961) was the highest salinity tolerated by *T. tubifex* without gradual acclimatization. Above this concentration worm deaths occurred in 24 hours. In the present study, NaCl at 0.1 M, the only concentration above 0.05 M tested, caused deaths among unoxygenated control worms also. Thus the highest salinity tolerated by *T. tubifex* corresponds closely to the saline concentration giving maximum protection against hyperbaric oxygen and the other stressing agents employed in the present study.

Palmer (1968) suggested that in higher salinities *T. tubifex* might be expected to require less energy expenditure to maintain itself hypertonic than in dilute environments causing continual tissue dilution. Such an argument could also be applied to explain the present findings of solute protection during oxygen poisoning. Palmer's experiments (1968) however did not support her hypothesis; she found no change in the oxygen consumption of *T. tubifex* in environments from zero to 20 per cent sea water.

The rather narrow concentration range for maximum protection indicates that the osmotic concentration of the environmental medium is an important factor in the response of *T. tubifex* to oxygen. The protective effect of added ions appears to have little specificity and to be independent of the ionic composition of the salt. The enhanced survival observed in these experiments following oxygen exposure of *T. tubifex* in the presence of ions or glucose indicates that almost complete protection can be obtained against these doses of oxygen in this organism. The protection provided by NaCl even when it is added some time after oxygen exposure indicates that in *T. tubifex* the effect of oxygen is at least partially reversible. The enhanced survival of *T. tubifex* in 0.05 *M* NaCl after heating or exposure to hydrogen peroxide suggests that increased salinity may be effective in protecting this organism against other environmental stresses.

I wish to express my deepest appreciation to Dr. Howard S. Ducoff for his advice and guidance throughout this study, to Dr. John D. Anderson for his continued interest and to the Department of Physiology and Biophysics for the use of their facilities.

SUMMARY

1. One per cent glucose when present during oxygen exposure provided significant protection of *T. tubifex* from doses which resulted in high worm mortality.

2. Glucose protection was not reversed by 2,4-dinitrophenol; 2,4-dinitrophenol was itself protective.

3. Sodium chloride at a concentration osmotically equivalent to one per cent glucose provided protection equal to or better than that of glucose.

4. Proteose-Peptone, $CaCl_2$, KCl, $CoCl_2$, $MnCl_2$ and $NaNO_3$ also provided various degrees of protection when they were present during oxygen exposure.

5. The solute concentration providing maximum protection against oxygen poisoning had an optimum at a concentration osmotically equivalent to one per cent glucose.

6. Sodium chloride increased the survival of oxygen-exposed *T. tubifex* even when it was added as long as 12 hours after oxygen treatment.

7. Sodium chloride provided at least partial protection against the stress of heating or hydrogen peroxide exposure.

LITERATURE CITED

Dickens, F., 1946a. The toxic effects of oxygen on brain metabolism and on tissue enzymes. 1. Brain metabolism. *Biochem. J.*, **40**: 145–171.

Dickens, F., 1946b. The toxic effects of oxygen on brain metabolism and on tissue enzymes. 2. Tissue enzymes. *Biochem. J.*, **40**: 177–187.

Freese, E., E. Bautz-Freese and E. Bautz, 1961. Hydroxylamine as a mutagenic and inactivating agent. *J. Mol. Biol.*, **3**: 133–143.

GERSCHMAN, R., D. L. GILBERT AND D. CACCAMISE, 1958a. Effect of various substances on survival times of mice exposed to different high oxygen tensions. *Amer. J. Physiol.*, **192**: 563–571.

GERSCHMAN, R., D. L. GILBERT AND J. N. FROST, 1958b. Sensitivity of *Paramecium caudatum* to high oxygen tensions and its modification by cobalt and manganese ions. *Amer. J. Physiol.*, **192**: 572–576.

HORN, R. S., C. D. WILLIAMS, E. S. HAUGAARD AND N. HAUGAARD, 1967. Toxic effects of oxygen on carbohydrate metabolism. *Fed. Proc.*, **26**: 709.

LOOMIS, W. F., AND F. LIPMANN, 1948. Reversible inhibition of the coupling between phosphorylation and oxidation. *J. Biol. Chem.*, **173**: 807–808.

PALMER, M. F., 1968. Aspects of the respiratory physiology of *Tubifex tubifex* in relation to its ecology. *J. Zool. London*, **154**: 463–473.

PROSSER, C. L., 1961. Water: Osmotic Balance. Pages 6–56 in C. L. Prosser and F. A. Brown Jr., Eds., *Comparative Animal Physiology*. [2nd. Edition] W. B. Saunders Co., Philadelphia.

SAIER, F. L. AND A. C. GIESE, 1967. The effect of ultraviolet radiation upon osmoregulation in Paramecium. *Photochem. Photobiol.*, **6**: 745–755.

WALKER, J. G., 1970. Oxygen Poisoning in the Annelid *Tubifex tubifex*. I. Response to oxygen exposure. *Biol. Bull.*, **138**: 235–244.

WITTNER, M., 1957. Inhibition and reversal of oxygen poisoning in *Paramecium*. *J. Protozool.*, **4**: 25–29.

Reference: *Biol. Bull.*, **140**: 166–189. (February, 1971)

THE LARVAL AND POSTLARVAL DEVELOPMENT OF *PARTHENOPE SERRATA* REARED IN THE LABORATORY AND THE SYSTEMATIC POSITION OF THE PARTHENOPINAE (CRUSTACEA, BRACHYURA)[1]

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According to Rathbun (1925), *Parthenope (Platylambrus) serrata* (H. Milne Edwards) has a range extending from the Bermudas, and Cape Hatteras, North Carolina, through the Gulf of Mexico and Bahama Islands, to Bahia, Brazil. The known substrate on which it occurs consists of sand, broken shells, gravel, corals or combinations of these. Holthuis (1959) found ovigerous females in Surinam from May to June. Williams (1965), citing the United States National Museum records, said ovigerous females have been collected during June in North Carolina, summer in Florida, and October in Cuba.

The studies of larval development of parthenopid crabs have been limited to the first zoeal stage or planktonic materials, and have dealt mostly with Mediterranean species. Gourret (1884) first mentioned the zoeae of *Parthenope massena* (Roux) (which he indicated with the name *Lambrus massena*). Cano (1893) described and illustrated three zoeal stages and Boraschi (1921) illustrated a telson of *Parthenope* species (as *Lambrus* sp.). Bourdillon-Casanova (1960) described and illustrated the first two zoeal stages of *P. massena* (as *L. massena*). Heegaard (1963) hatched and described the first zoeal stages of *P. massena* and *P. angulifrons* Latreille (as *L. massena* and *L. angulifrons*), presenting a color plate to show the chromatophore patterns. Aikawa (1937) described the first zoea of the Japanese species *P. valida* De Haan (as *L. validus*) reared from hatching. The present work is the first study of the complete larval and postlarval development of any species of the Parthenopidae, based on reared animals.

Since Milne Edwards' (1834) definition of family Oxyrhinques (=Oxyrhyncha), all major workers of Oxyrhyncha (*e.g.*, Dana, 1851; Miers, 1879; Alcock, 1895; Rathbun, 1925; Sakai, 1938; Balss, 1957; Garth, 1958) have placed the Parthenopidae under Oxyrhyncha.

MATERIALS AND METHODS

Two ovigerous females used in the present work were collected by a Biscayne Bay shrimp trawler on 4 March, 1965. These were kept in a running sea-water aquarium with sand. When eye spots appeared in the eggs each ovigerous female was then placed into a 25-cm diameter finger bowl of clear plastic with filtered

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Biscayne Bay water. The females were fed cut shrimp and the water was changed daily. Hatching occurred on 13 March and 4 April, 1965.

After hatching, each larva was coded with a serial number and placed singly in a compartment of a plastic culture box. Each hatch consisted of 90 larvae reared at 25° C; 35 larvae from the first brood and 45 larvae from the second brood were reared at 20° C. Each compartment contained about 20 cc of filtered Biscayne Bay water. The first three zoeal stages were fed the smallest nauplii of just-hatched Artemia eggs, obtained by filtering through stainless steel screen (105 × 105 mesh, 0.0003 inch wire). The later zoeal and megalopal stages were fed unfiltered newly hatched *Artemia*. Chopped fresh shrimp was fed to the early stage crabs. Animals were changed to compartments with clean seawater, fed fresh food daily and were checked for molted and dead individuals. Salinity range of the rearing seawater was 34–37‰.

Exuviae and specimens of each developmental stage were preserved in 7% buffered formalin. These were stained with Mallory's acid fuchsin red or chlorazol black. The larval appendages for drawing were dissected in 85% lactic acid, while crab appendages were dissected in full strength ethylene glycol. The appendages after drawing were mounted permanently in Turtox CMCS, a stained water miscible mounting medium. Drawings were made with aid of a camera lucida. Details of the appendages were checked under high power (400 × or more). Four specimens of each stage were measured with a calibrated ocular micrometer. The carapace length of zoeae was measured in lateral profile from the anterior margin of the ocular peduncle to the extremity of the posterior margin of the carapace. Carapace length of megalopa and crab were measured dorsally from the tip of the rostral spine to the posterior margin of the carapace, and the width was measured across the widest part of the carapace. The size given for each stage is the arithmetic average of four specimens examined. The formulae for order of setation denote the number of setae from the proximal to the distal segment (or group) and was also based on four specimens.

The female crabs are deposited in the museum of the Institute of Marine and Atmospheric Sciences of the University of Miami (UMML 32-3356 and UMML 32-3582).

Morphological Results

The number of zoeal stages through which *Parthenope serrata* passes to reach the megalopa is five or six. Most individuals surviving to the crab stage had passed through six zoeal stages. The text figures provided are as accurate as possible, hence the text is mainly restricted to comments on features not obvious from the figures or to emphasize any variations that were found.

First zoea (Fig. 1)

Carapace: Length approximately 0.37 mm. Dorsal, rostral and lateral spines as illustrated. Forehead rounded, with muscle bands plus a small protuberance. No anterior seta, nor infero-lateral setae present. A seta on each postero-lateral part of dorsal spine. Eye sessile with minute ocular papillae. *Abdomen*: Lateral knobs, postero-lateral process as shown. *Telson*: Lunate, bearing a dorsal spine

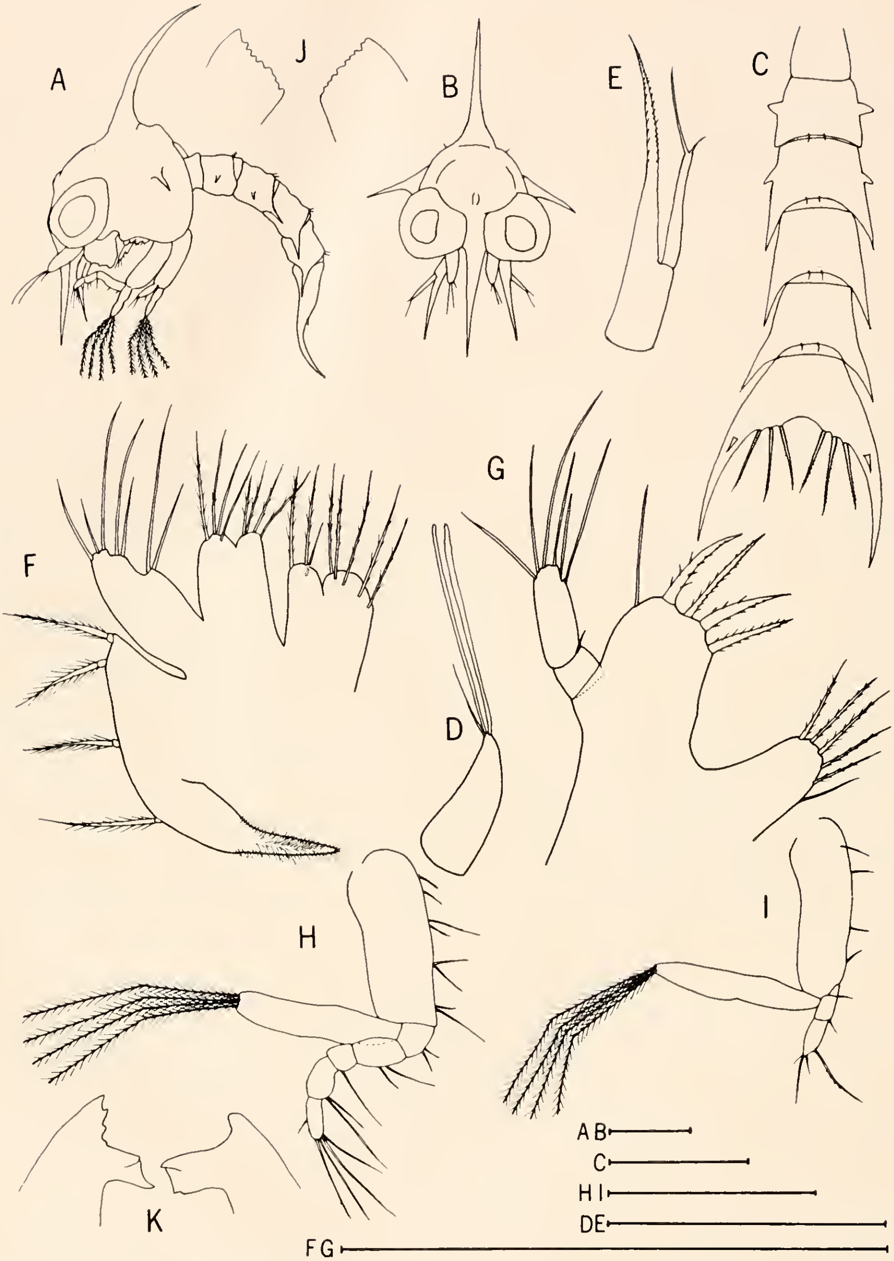

FIGURE 1. *Parthenope serrata*: First zoea; A, lateral view; B, anterior view of carapace; C, abdomen; D, antenna 1; E, antenna 2; F, maxilla 2; G, maxilla 1; H, maxilliped 1; I, maxilliped 2; J, antero-ventral view of mandible; K, postero-median view of mandible. Bar scales represent 0.2 mm.

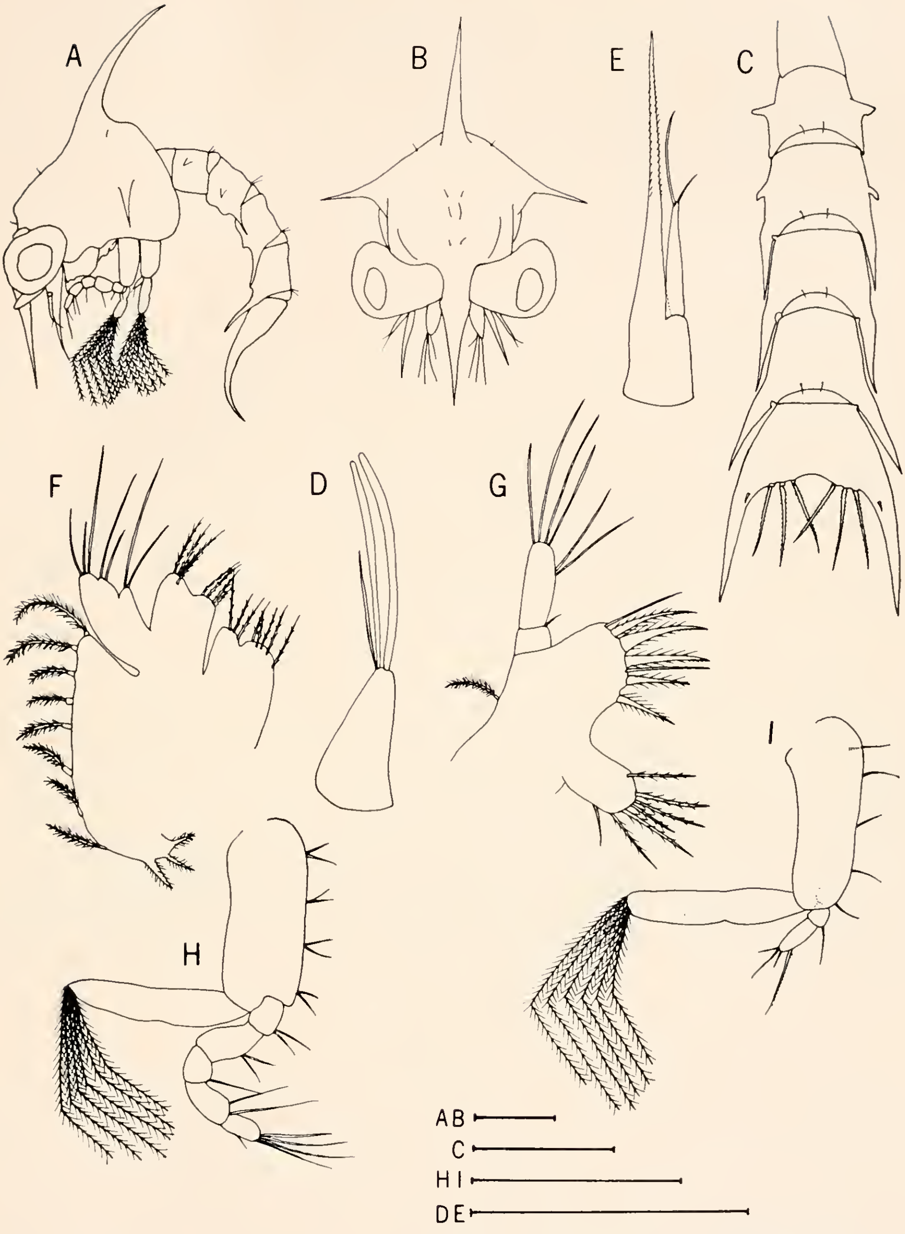

FIGURE 2. *Parthenope serrata*: Second zoea; A, lateral view, B, anterior view of carapace; C, abdomen; D, antenna 1; E, antenna 2; F, maxilla 2; G, maxilla 1; H, maxilliped 1; I, maxilliped 2. Bar scales represent 0.2 mm.

on each fork. Occasionally a minute setule present on lateral margin of telson halfway to fork, visible only under high magnification (400 ×). *Mandible*: Nine to ten teeth on antero-ventral margin, three teeth on right postero-medial margin (none on left). *Maxilliped 1*: Endopodite, five-segmented, setae arranged (2, 2, 1, 2, 5). *Color*: Melanophores located on dorsal basal area of each lateral spine; on posterior basal part of dorsal spine; postero-ventral portion of carapace; on each mandible; on each side of the ventral surface of abdominal somites 1–5; on basal part of antenna 2. Inconspicuous melanophore located on interorbital area of rostrum and at middle part of basipodite of maxilliped 1. Eye a light yellow-green. *Pereiopods:* Buds present, minute.

Second zoea (Fig. 2)

Carapace: Length approximately 0.48 mm. Eyes stalked. In subsequent stages dorsal spine continually shortens in relation to carapace length and anterior base thickens. Four setae added on forehead, plus five hairs along infero-lateral margin. *Abdomen:* Lateral process of somites 3–5 more elongated. *Telson:* Inner margin less lunate than zoea 1. Dorsal spine on fork now reduced. *Maxilla 1:* A plumose seta added to (proximal to) endopodite. Basal and coxal endites each with increased number of setae. *Maxilla 2:* Scaphognathite setae increased to approximately eight; apical process now divided into three. *Maxillipeds 1 and 2:* Now with six natatory setae.

Third zoea (Fig. 3)

Carapace: Length approximately 0.59 mm. Forehead and anterior base of dorsal spine nearly a straight line. Additional setae added below forehead protuberance. Groove forming in proximal portion of rostral spine between eyes. *Abdomen:* Somite 6 now separated from telson. A pair of mid-dorsal setae on somite 1 (one seta in some individuals). *Antenna 1:* One small aesthetasc added. *Antenna 2:* Small endopodite bud appears. *Maxilla 1:* Basal endite now with nine setae. *Maxilla 2:* Space between three processes of endopodite noticeably increased. Scaphognathite with approximately 10 setae in addition to apical processes. *Maxillipeds 1 and 2:* Eight natatory setae. *Maxilliped 1:* Setae now arranged (2, 2, 1, 2, 6).

Fourth zoea (Fig. 4)

Carapace: Length approximately 0.66 mm. Lateral spines further reduced. Rostral spine at interorbital area well grooved. *Abdomen:* Somite 1 usually with three mid-dorsal setae. *Telson:* Dorsal spine on fork now minute. *Antenna 2:* Endopodite bud elongated. *Maxilla 2:* Scaphognathite usually with 14 plumose setae plus three to five divided apical processes. *Maxillipeds 1 and 2:* Eight and nine natatory setae, respectively. Occasionally these numbers reversed; or rarely eight on both. *Pereiopods:* Buds elongated and easily seen. *Pleopods:* Bud primordia recognizable.

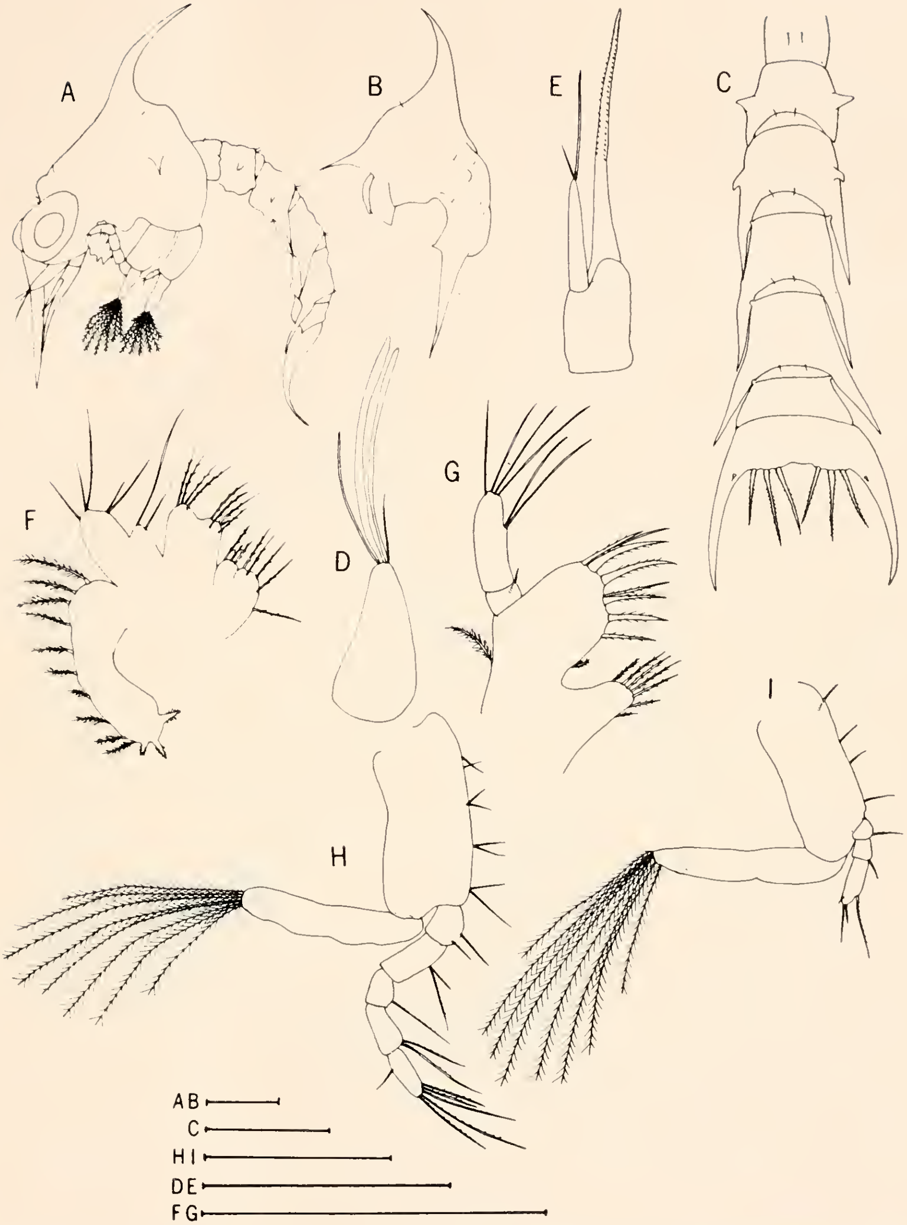

FIGURE 3. *Parthenope serrata*: Third zoea; A, lateral view, B, antero-lateral view of carapace; C, abdomen; D, antenna 1; E, antenna 2; F, maxilla 2; G, maxilla 1; H, maxilliped 1; maxilliped 2. Bar scales represent 0.2 mm.

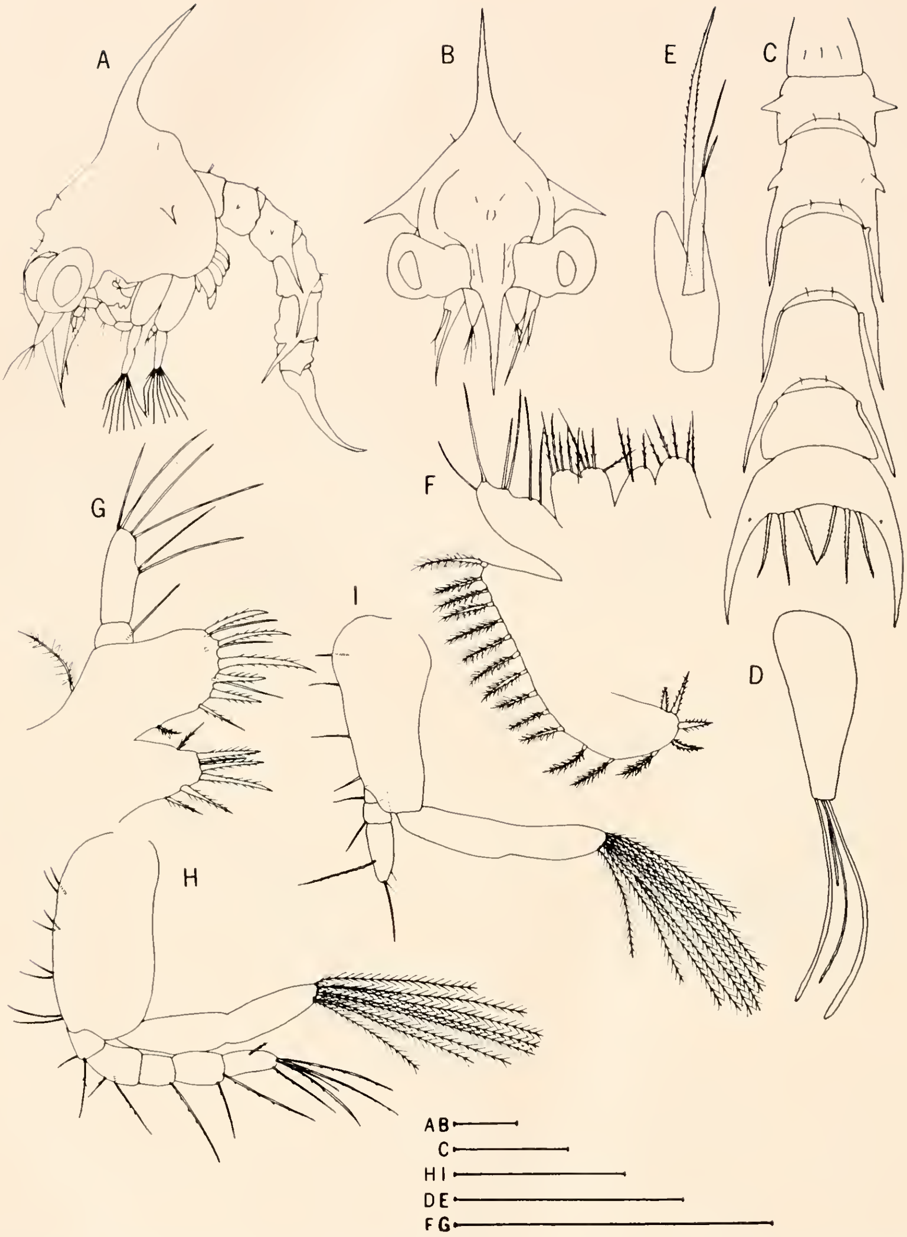

FIGURE 4. *Parthenope serrata*: Fourth zoea; A, lateral view; B, antero-lateral view of carapace; C, abdomen; D, antenna 1; E, antenna 2; F, maxilla 2; G, maxilla 1; H, maxilliped 1; I, maxilliped 2. Bar scales represent 0.2 mm.

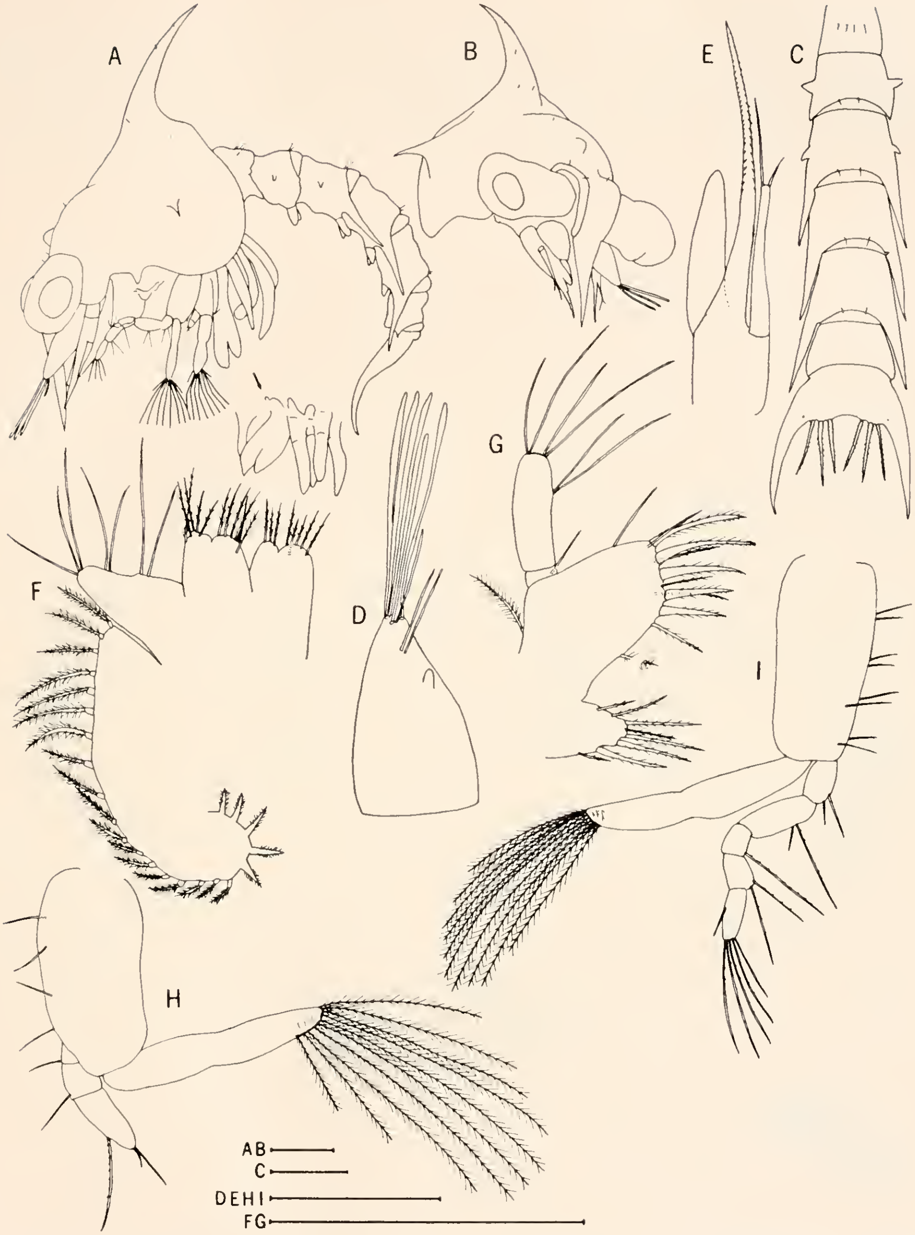

Figure 5. *Parthenope serrata*: Fifth zoea; A, lateral view; B, antero-lateral view of carapace; C, abdomen; D, antenna 1; E, antenna 2; F, maxilla 2; G, maxilla 1; H, maxilliped 1; I, maxilliped 2. Bar scales represent 0.2 mm.

Fifth zoea (*Fig. 5*)

Carapace: Length approximately 0.79 mm. General form more depressed than previous stages along with more stout and shorter dorsal spine. Two small setae on infero-lateral margin; a few minute setae posterior to these on future branchiostegite membrane. *Abdomen:* Somite 1 with approximately four

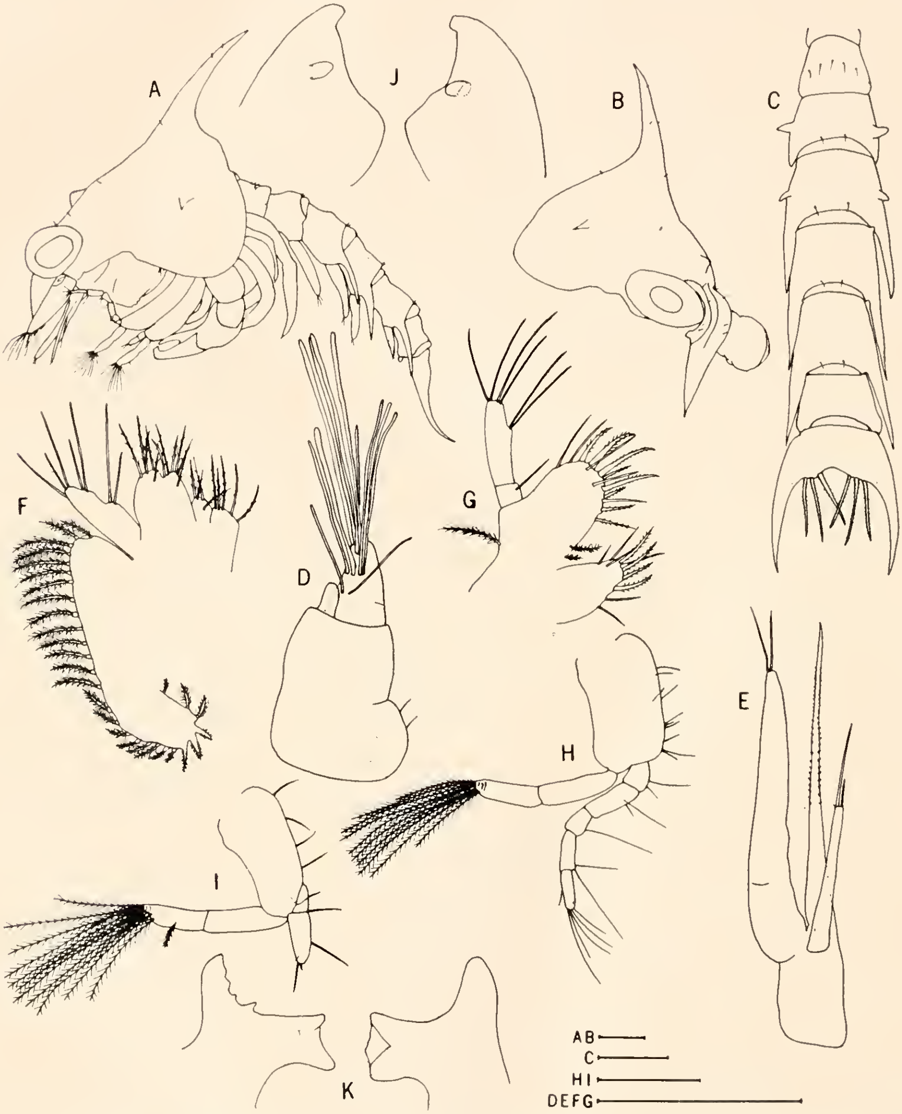

FIGURE 6. *Parthenope serrata*: Sixth zoea; A, lateral view; B, antero-lateral view of carapace; C, abdomen; D, antenna 1; E, antenna 2; F, maxilla 2; G, maxilla 1; H, maxilliped 1; maxilliped 2. Bar scales represent 0.2 mm.

dorsal setae. *Telson:* Dorsal spine on fork now rudimentary. *Antenna 1:* Six terminal, two (rarely one) subterminal aesthetascs. Distal position anticipates segmentation. Inner flagellum bud appears on medial margin. *Antenna 2:* Endopodite bud now extends beyond exopodite. *Maxillipeds 1 and 2:* Usually ten natatory setae each. *Pereiopods:* Buds enlarged, chelation beginning. *Pleopods:* Buds much elongated. *Gills:* Buds now visible.

Sixth zoea (*Fig. 6*)

Carapace: Length approximately 1.01 mm. More depressed; dorsal spine stouter, inclined more posteriorly than previous stage. Lateral spine much shorter. Rostrum thickened, with median ridge on proximal portion plus two setae. *Abdomen:* With approximately five mid-dorsal setae on somite 1. *Antenna 1:* Peduncle, inner flagellum, and dorsal flagellum differentiated. Partly segmented peduncle with a dorsal indentation. Dorsal flagellum with three groups of aesthetascs placed (5, 5, 2). *Antenna 2:* Endopodite remarkably elongated plus two terminal setae; anticipating segmentation. *Mandible:* Anterior proximal portion with a palp bud. *Maxilla 1:* Form remains unchanged from second zoeal stage but setae increase in number. *Maxilla 2:* Scaphognathite with approximately 23 setae; with branched apical processes. *Maxilliped 1:* Ten natatory setae. Basipodite setae increased (2, 2, 3, 3). *Maxilliped 2:* Eleven natatory setae. *Pereiopods:* Buds well developed. Chelae almost formed. *Pleopods:* Elongated, with terminal setae. *Gills:* Buds continue forming in respective locations; in exuviae they appear to be laminated.

Megalopa (*Figs. 7, 8*)

Carapace: Length approximately 1.47 mm; width 0.98 mm. Medial portion of rostrum at interorbital region slightly depressed. Gastric region moderately inflated and without protruding ridges as in majiid megalopae. Hepatic lobe a hemisphere. Cardiac spine (dorsal spine in zoeal stages) with few minute setae. *Abdomen:* Six somites plus telson; setation as shown. *Antenna 1:* Peduncle three-segmented; dorsal portion of proximal segment concave, supporting proximal portion of eye peduncle with expanded lateral margin. Segmentation between distal and penultimate segments of outer flagellum unclear. *Antenna 2:* No modifications on distal portion of basal article (such as in majiid megalopae). *Mandible:* Now with two-segmented palp. *Maxilla 2:* Apical processes of scaphognathite now bushy plumose setae, similar in form to marginal setae. A few setae on dorsal and ventral surface of scaphognathite. Endopodite with added lateral plumose setae. *Maxilliped 1:* Shape now radically changed; epipodite now fringed with seven or more smooth hairs. *Maxilliped 2:* Epipodite bud with one or two terminal setae. *Maxilliped 3:* As illustrated. *Pereiopods:* Sparsely covered with setae. Right cheliped slightly larger than left. Dactyl of pereiopod 5 with long subterminal hair (=feeler); hair tip hook-shaped. *Pleopods:* Present on somites 2–6, natatory setae arrangement on exopodite progressing distally as follows: 13, 14, 13, 10, 4. Numbers of natatory setae inconsistant, varying considerably between specimens, and on different sides of same individual. Appendix interna usually with three hooks.

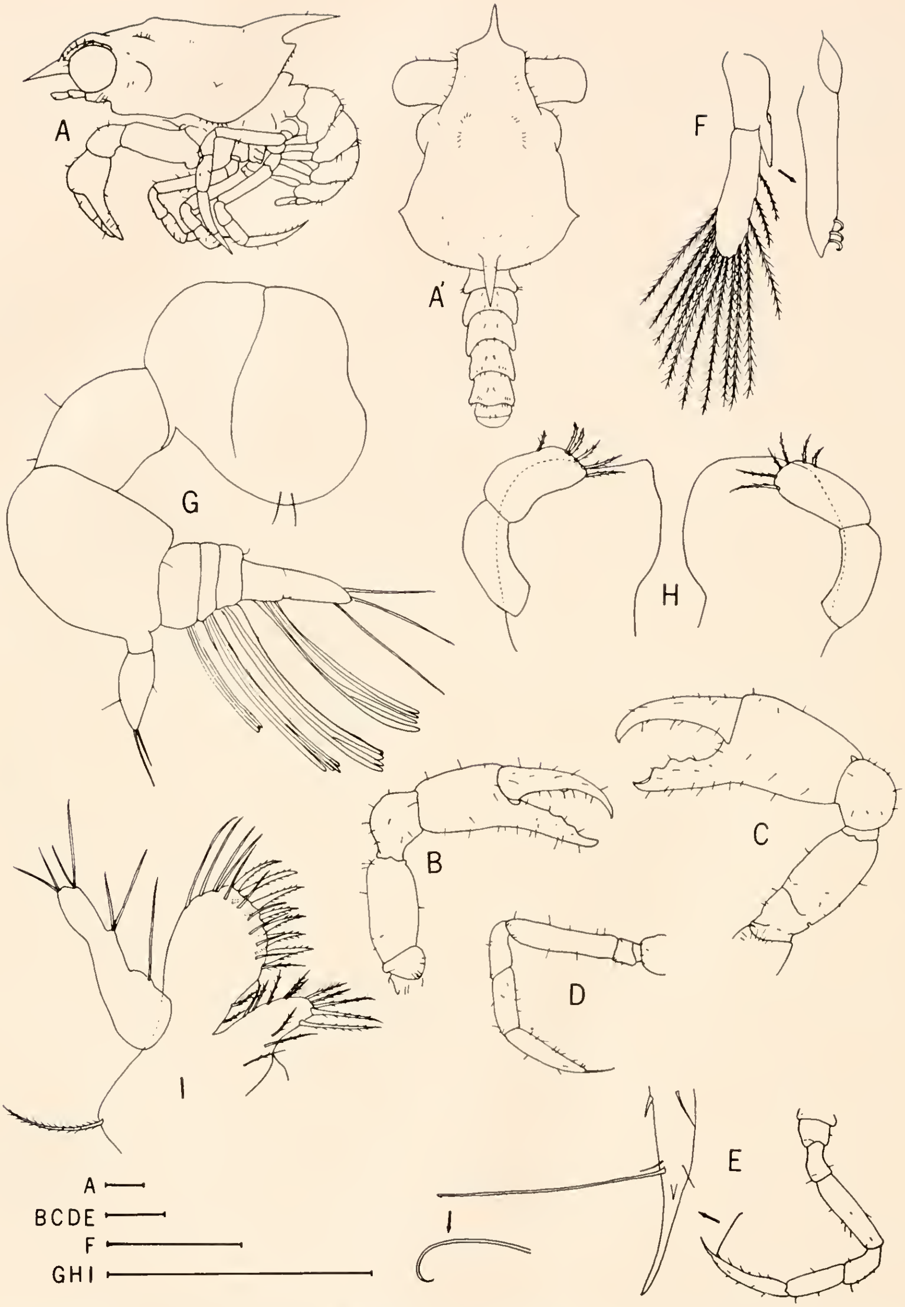

FIGURE 7. *Parthenope serrata*: Megalopa A, lateral view; A, dorsal view; B, left cheliped; C, right cheliped; D, pereiopod 2; E, pereiopod 5; F, pleopod; G, antenna 1; H, mandible; I, maxilla 1. Bar scales represent 0.2 mm.

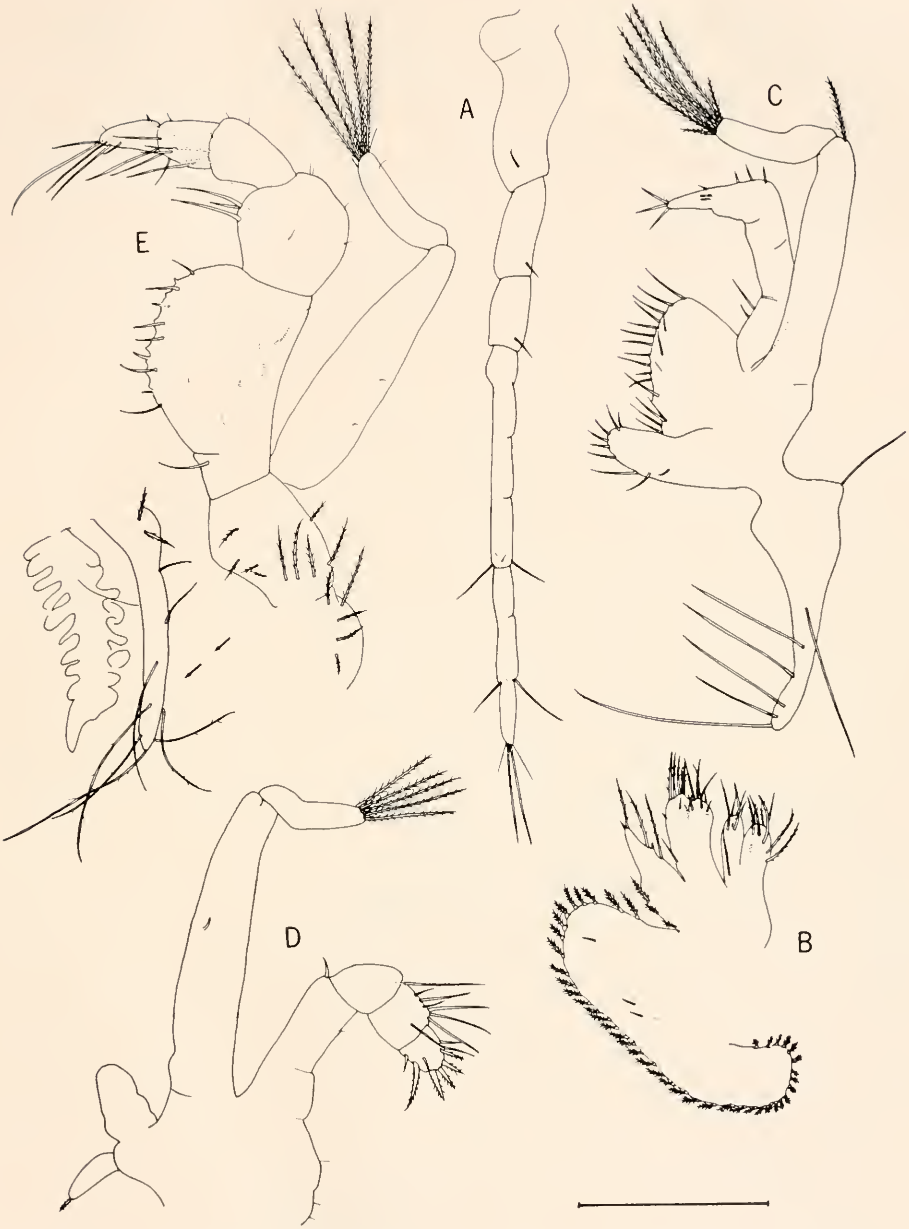

Figure 8. *Parthenope serrata*: Megalopa; A, antenna 2; B, maxilla 2; C, maxilliped 1; D, maxilliped 2; E, maxilliped 3. Bar scale represents 0.2 mm.

Gills: Development more advanced than in majiid megalopae. Maxilliped 2 with one elongate bud of future podobranch, incipient epipodite with two minute distal hairs. Maxilliped 3 with well laminated posterior arthrobranch, elongate

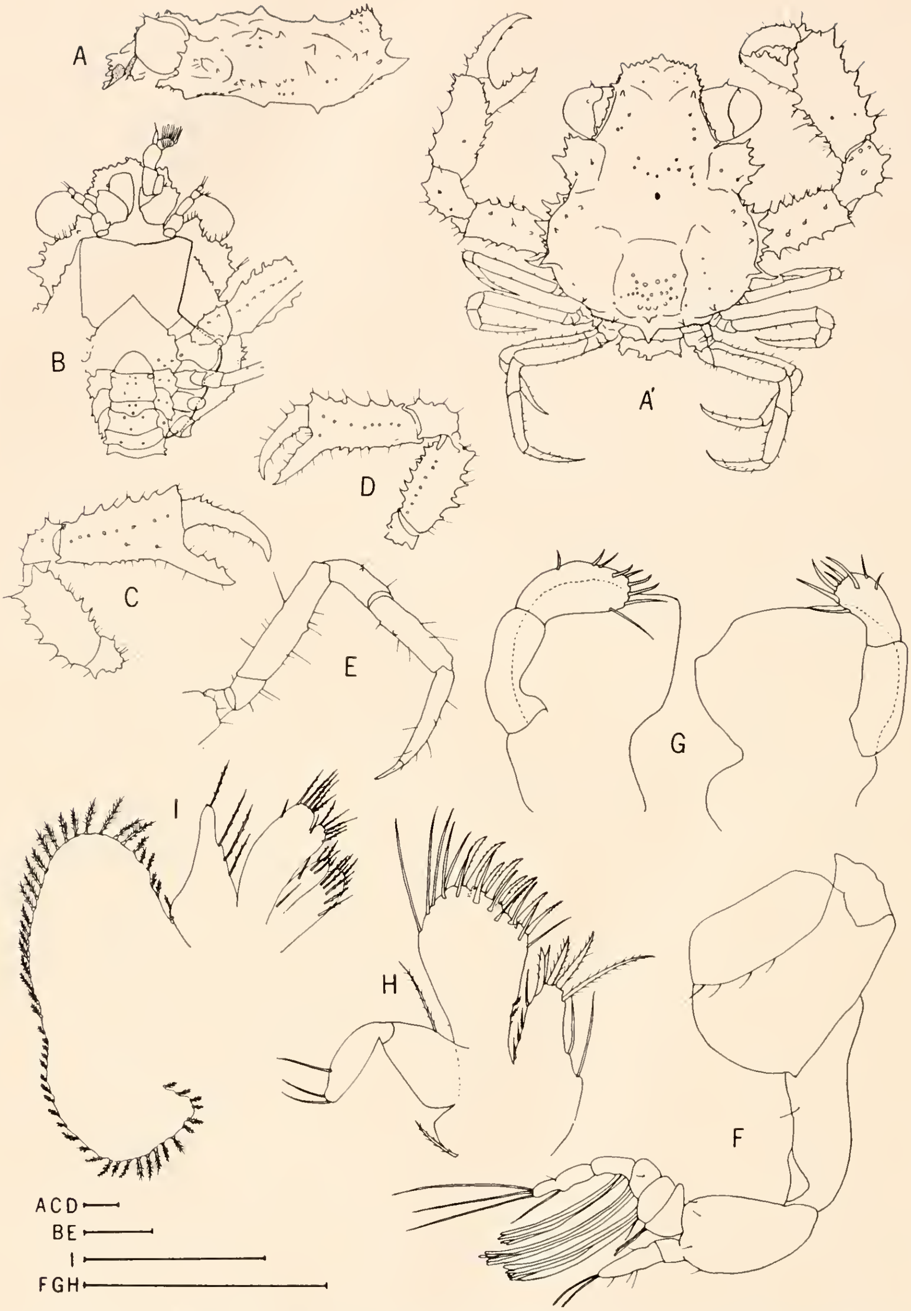

FIGURE 9. *Parthenope serrata*: First crab; A, lateral view of carapace; A′, dorsal view; B, ventral view of carapace; C, ventral view of right cheliped; D, ventral view of left cheliped; E, pereiopod 5; F, antenna 1; G, mandible; H, maxilla 1; I, maxilla 2. Bar scales represent 0.2 mm.

bud of anterior arthrobranch, and small bud of podobranch. Cheliped with two well laminated arthrobranchs. Pereiopods 2 and 3 each with a pleurobranch.

Color: Carapace shaded pale yellow. Melanophores on carapace and appendages placed as follows: stippled over dorsal surface of carapace; on proximal portion of rostrum at interorbital area; on posterior base of cardiac spine; on dorsal and inferior side of each branchial postero-lateral spine; on peduncle of eye; on first segment of peduncle of antenna 1; on coxopodite, meropodite and propodite of pereiopods 2–5; and on ischiopodite of maxilliped 3. Melanophores on cheliped as follows: two at base of hiatus, two at proximal portion of propodite, one at carpopodite, three small ones at meropodite; paired on ventral side of abdominal somites 2–6. Basipodite of pleopods on somites 2–6 and telson each with a faint melanophore and red chromatophore.

First Crab (*Figs. 9, 10*)

Carapace: Length approximately 1.70 mm; width 1.48 mm. Depth of carapace at cardiac region slightly greater than at gastric region. Medial portion of rostrum depressed into groove. *Abdomen:* Seven-segmented. No marked locking mechanism between it and thoracic sternum. *Antenna 1:* Dorsal surface of peduncle cup-shaped, well calcified with several hairs, acting as ventral floor of eye stalk. *Antenna 2:* Flagellum less calcified than peduncle, with seven segments. *Maxilla 1:* Endopodite segmentation obscure. Plumose seta from second zoeal stage still present on lateral margin, proximal to endopodite. *Maxilla 2:* Scaphognathite now with approximately 52 plumose setae. *Maxilliped 1:* Endopodite hatchet-shaped, apparently one-segmented. *Maxilliped 2:* Epipodite still small with two terminal hairs. *Maxilliped 3:* Ischio-basipodite cleavage slightly marked, with serrated ischiopodite medial margin. *Pereiopods:* Lateral margins of cheliped strongly serrated as in adult. Right cheliped, as in megalopa, much larger than left. *Pleopods:* Buds of subsequent crab stage pleopods 2–5 apparently present, but hard to detect.

Gills: Appear on appendages as follows: Maxilliped 2 with elongated bud of epipodite bearing one or two terminal hairs, and elongated podobranch bud. Bud of arthrobranch not apparent in this stage but is observed in second crab stage. Maxilliped 3 with well laminated posterior arthrobranch, and slightly laminated bud of podobranch and anterior arthrobranch. Cheliped with two well laminated arthrobranchs. Pereiopods 2 and 3 each with a pleurobranch.

Color: Minute melanophores quite densely spotted on carapace, and on third maxilliped. Chelipeds, pereiopods 2–5, and proximal portion of antennae with small melanophores. Paired melanophores on external side of abdominal somites 2–6.

REARING RESULTS

Two broods from different ovigerous females collected at the same time and locality have been reared. Although the morphological study was based on material from the two broods, the present rearing results are of the brood which hatched on 4 April 1965. An ovigerous female (carapace length 17.2 mm; carapace width including lateral spines 24.5 mm) produced approximately 3900 zoeae in one brood.

Figure 11 illustrates survival and mortality of the animals reared at 25° C including the individuals which molted into megalopa after five zoeal stages. A little less than one half of the initial population molted into second zoea. After

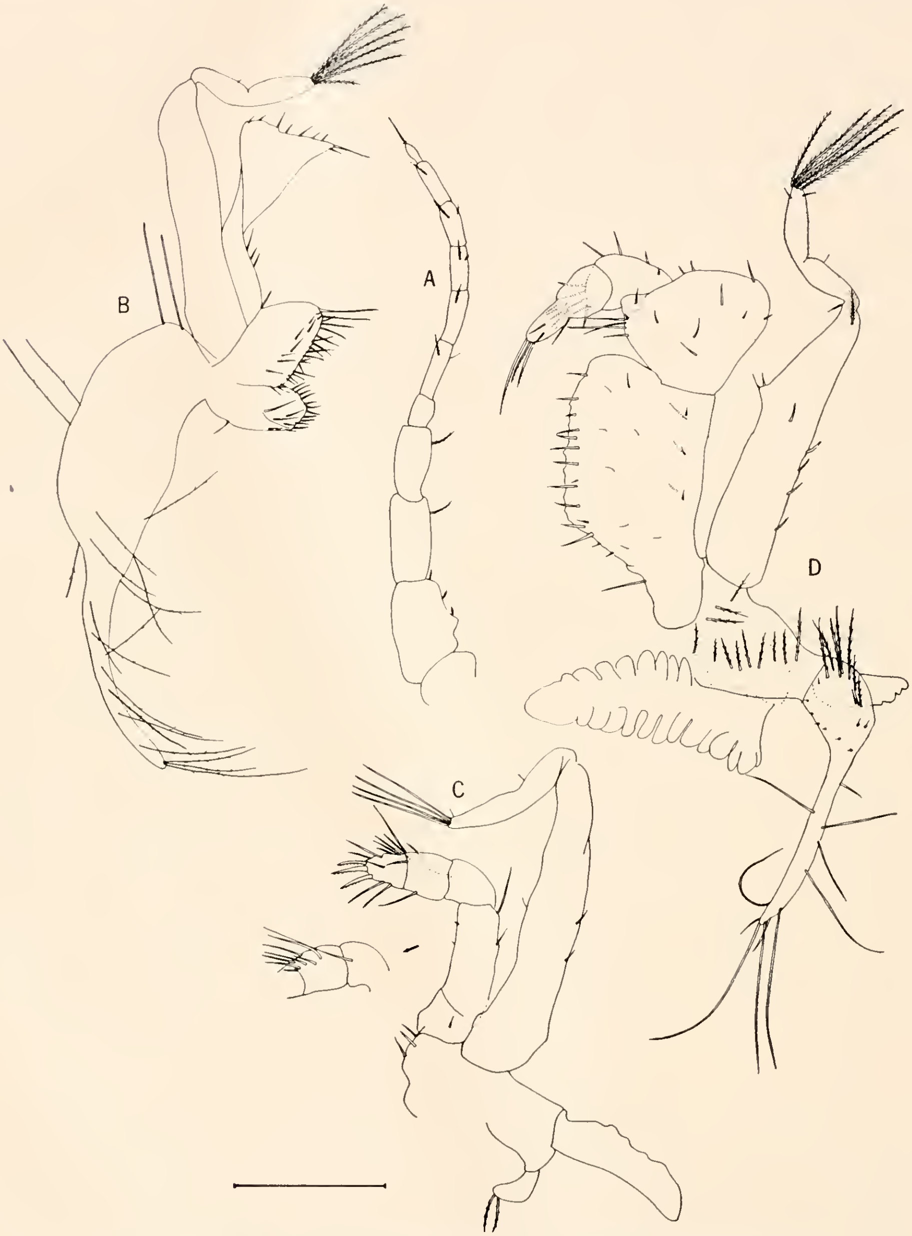

FIGURE 10. *Parthenope serrata*: First crab; A, antenna 2; B, maxilliped 1; C, maxilliped 2; D, maxilliped 3. Bar scale represents 0.2 mm.

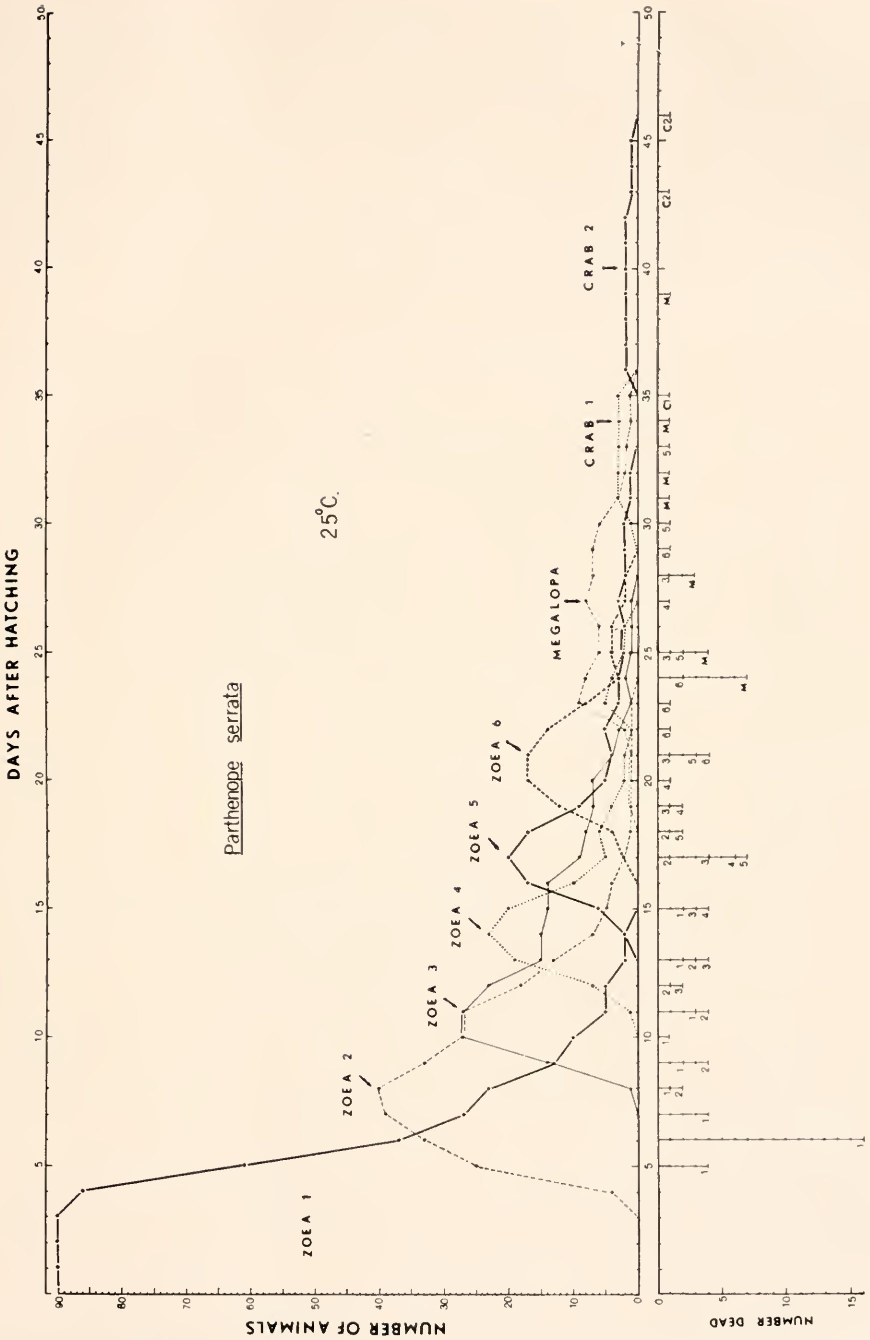

Figure 11. *Parthenope serrata*: rearing record at 25° C. The horizontal scale represents days after hatching. The vertical scale represents number of animals. The lower figure indicates number of deaths per day. The symbols 1 to 6, M, c1 and c2 represent, respectively, zoea 1 to zoea 6, megalopa, crab 1 and crab 2 stages.

the second zoeal stage, the number of individuals surviving into succeeding stages gradually decreased. Approximately one-ninth of the initial population reached the megalopa stage, but mortality in the megalopa stage was high. In this rearing none of the megalopae which had passed through five zoeal stages molted into the first crab stage. Three individuals out of the initial zoeal population of 90 reached the first crab stage in about 30 days after hatching and two individuals developed into the second crab stage. Among the larvae of this brood reared in 20° C two individuals reached the first crab stage in about 45 days; one individual reached the second crab stage but died immediately after molting.

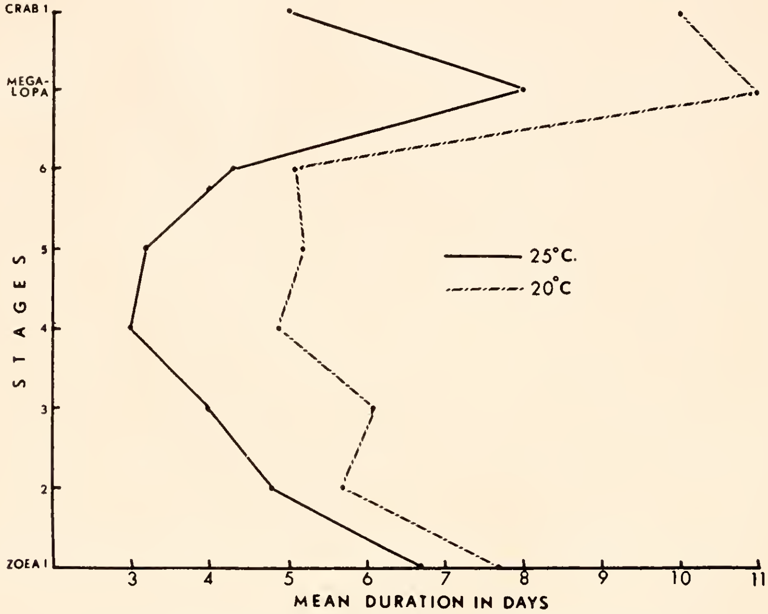

FIGURE 12. Duration of the larval and post-larval stages of *P. serrata* under two different temperatures.

The mean duration of each stage at 20 and 25° C is shown in Figure 12. The data of individuals reared at 20° C are based on an initial zoeal population of 45 individuals. The duration of the first crab stage at 20° C rearing is based on a single individual. The first zoeal stage at both temperatures had a greater duration than the succeeding stage. The majority of individuals which had a very prolonged first zoeal stage failed to complete development although some reached the intermediate zoeal stages. The general pattern of mean duration is quite similar in both 20° and 25° C although mean duration of intermediate zoeal stages was longer at 20° C than at 25° C. A prolonged duration of the megalopa stage is well marked in both groups. Provenzano (1968) observed a similar pattern in a study of anomuran larvae, and suggested that the prolongation of the metazoea and megalopa was probably due to reorganization of body structure. Robertson (1968) found a similar though less marked trend in his

TABLE I

Comparison of characters of first zoeal stage of Parthenopidae of known parentage

Species	*Parthenope serrata* H. Milne Edwards	*P. valida* De Haan	*P. massena* (Roux)	*P. angulifrons* Latreille
Reference	Present work	Aikawa, 1937	Heegaard, 1963	Heegaard, 1963
Dorsal spine	Much longer than carapace depth, tapers distally, bends posteriorly	Almost equal to carapace depth, tapers distally, bends posteriorly	Much longer than carapace depth, tapers distally, bends posteriorly	Much longer than carapace depth, stout, and blunt, somewhat straight
Rostral spine	Long, pointed	Long, pointed	Long, pointed	Long, pointed
Lateral spine	Fairly long	Short (?)	Fairly long	Fairly long
Postero-lateral processes on somites 3–5	Elongated projection, pointed	No projection, not pointed	Elongated projection, pointed	No projection, not pointed
Telson	Lunate	Lunate	Lunate	Slightly lunate
Spine on telson fork	1 dorsal spine only	1 dorsal only	1 dorsal spine, 1 lateral seta	1 dorsal spine, 1 lateral seta
Exopodite of antenna 2	1-segmented, 2 terminal spines	1-segmented, 2 terminal spines	2-segmented, 2 terminal spines	1-segmented, tapers distally with 2 subterminal spines
Endopodite of maxilla 1 (seta dist.) (seta prox.)	2-segmented, (2–4) (1)	2-segmented (2–4) (1)	3-segmented (2–4) (0)	3-segmented (2–4) (0)
Medial margin of endopodite of maxilla 2 (setae)	3 processes (2,2,3)	2 processes (?) (2,3+2)	3 processes (2,1,2)	2 processes (2,4)
Endopodite maxilliped 2	3-segmented	3-segmented	1-segmented (?)	2-segmented (?)
Locality	Florida, U. S. A.	Japan	Mediterranean	Mediterranean

study of phyllosomas of the lobster, *Scyllarus americanus* (Smith). The marked prolongation of the megalopa stage has been seen in the larval development of majid crabs which have only two zoeal stages (Yang, 1967).

DISCUSSION

The number of zoeal stages in development of the Parthenopidae has been in question for a long period. After noting that Cano described three zoeal stages, Lebour (1928, page 555) predicted that "there probably are four or five." Bourdillon-Casanova (1960) also assumed that the Parthenopidae had more than two zoeal stages.

Table I is constructed from the major anatomical distinctions and similarities among *P. serrata* and three species of parthenopid first zoeae of known parentage reared by Aikawa (1937) and Heegaard (1963).

There are some apparent character differences among the four reared species of parthenopid zoeae. However, there are unique zoeal characters which allow the larvae to be identified as parthenopids. These are summarized from the four species plus *Parthenope* (*P.*) *agona* and *Solenolambrus typicus,* also reared by the author (Yang, unpublished):

1. The carapace has well developed dorsal, lateral and rostral spines.
2. The outline of the telson is lunate with a "dorsally located spine" on each fork. A minute hair-like lateral seta is sometimes present.
3. Lateral knobs are present on abdominal somites 2–3.
4. The forehead protuberance is well developed as in most majid zoeae and the postero-dorsal knob of the carapace also appears to be present in the majority of parthenopids studied.
5. The endopodite of maxilliped 2 appears to be three-segmented with a disto-medial seta on the two proximal segments.

The carapace of the first zoeae is more or less spherical with long dorsal, rostral, and lateral spines. As zoeae molt to the subsequent stages, the distance between the anterior part of the eye and the basal part of the dorsal spine increases. The length of the dorsal and lateral spines rapidly decreases in proportion to the carapace size as does the rostral spine, while carapace height is also reduced at each subsequent stage. The dorsal spine thickens basally and shifts posteriorly. Thus, the distance between the posterior base of the dorsal spine and the postero-dorsal margin of the carapace decreases. At the sixth (last) zoeal stage, the carapace is remarkably depressed with shorter dorsal and reduced lateral spines and broader rostral spines. Thus, the carapace form becomes similar to that of the megalopa. A *Parthenope* megalopa retains the lateral spines of the zoeae on the branchial region. The so-called dorsal spine of the zoeal stage is a cardiac spine. This gradual change of shape is also seen in the zoeal stages and megalopa of *Callinectes* illustrated by Costlow and Bookhout (1959), and among other long-zoeae-stage crabs. Further, this shifting of the zoeal form into the megalopa is clearly observed in *Stenorhynchus seticornis* (Herbst) and *Rochinia hystrix* (Stimpson), both having but two zoeal stages (Yang, 1967).

Costlow (1965), and Rice and Provenzano (1966) reviewed the variability in larval stages of decapod larvae. In the present study, in one rearing of *P. serrata* at 25° C, zoeae developed into the megalopa stage. Thirteen of these molted from the fifth zoeae to megalopa while eleven molted from the sixth zoeae. Of the former, ten individuals died during or immediately after molting and three died later. Of those produced after 6 zoeal stages, two megalopae subsequently developed into crabs.

In the fourth zoeae of the five-staged series there are nine and ten natatory setae on maxillipeds 1 and 2, respectively. The fifth zoea of these series each have ten natatory setae on maxillipeds 1 and 2. The five-staged zoeae have a well-developed endopodite on antenna 2 and pleopods buds at the fifth stage, and the length is similar to the normal six-staged zoeae, but the development of antenna 1 appears to be poor without an inner-flagellum bud, and with only two rows of aesthetascs.

In normal six-staged zoeal development, the inconsistent number of the plumose natatory setae was mentioned earlier. This variability of number of natatory setae is also reported by Yatsuzuka (1957) for *Portunus pelagicus* (L., as *Neptunus pelagicus*) and five other species of crabs.

I studied the larval and postlarval development of more than twelve species of Majidae from hatching, and determined that the zoeae of the Parthenopidae, which have Brachyrhynchan zoeal characters, are easily distinguished from the majid zoeae in the following respects:

1. Six zoeal stages are observed in *P. serrata*, whereas in the true majid crabs the number of zoeal stages is apparently fixed at two, reflecting precocious or abbreviated development. The early zoeae of *P. serrata* and of the other Parthenopidae discussed above are alike in their anatomy, which is of a type characteristic for crabs with multi-zoeal development.
2. The zoeae of the Parthenopinae have dorsal, rostral, and lateral spines on the carapace. In general, dorsal and rostral spines are seen in the true majid zoeae. In the zoeae of the Inachinae (Majidae) only a dorsal spine is present.
3. The telson of the zoeae of the Parthenopinae is lunate with a spine located dorsally on each fork. In the Majidae this spine is lateral.
4. The exopodite of antenna 2 differs from that of general majid zoeae in having 2 terminal instead of 2 subterminal spines, plus a well developed endopodite bud in the first zoeal stage.
5. The "anterior seta" (Bourdillon-Casanova, 1960), present in the majid zoeae, is wanting in the zoeae of Parthenopinae.

Lebour (1928), discussing parthenopid zoeal characters, stated (page 555), "They are more like Cancridae, having all the carapace spines, antennae like *Portunus* (actually *Macropipus*), and only one lateral spine on the telson. If these larvae be correctly identified and representing the Parthenopidae, then this family does not agree with Majidae in any way, and is an exception among the Oxyrhyncha." Aikawa (1935) merely mentioned that the zoeae of the Parthenopidae are remarkably close to those of the Cancridae, and later he placed the zoeae of the Parthenopidae into a group of the Cancridae (Aikawa, 1937).

Although both parthenopid and cancroid zoeae share many zoeal features, the cancroid zoeae of known parentage described by Lebour (1928), Aikawa (1937), Fagetti (1960), Mir (1961), and Pool (1966) show a remarkably different type of telson compared to that of the Parthenopidae. The cancroid zoeae possess lateral-knobs only on abdominal somite 2.

Lebour (1944) gave a brief description and illustration of a megalopa which she attributed to a species of *Parthenope*, based on the elongated chelipeds and lack of "feelers" on the dactyl of pereiopod 5. She mentioned that her megalopa also had lateral spines on abdominal somites 3–5, and the pleopod on somite 6 bore numerous spines. There are no such features in *P. serrata*. The elongated rostral spine and the posteriorly elongated cardiac spine of megalopa she examined are somewhat similar to *P. serrata*.

Lebour (1928, page 490) stated, "The presence of feelers on the last joint of the last legs cuts off the Brachyrhyncha (with exception of Pinnotheridae) from the Oxyrhyncha and from *Ebalia*. . . ." Gamō (1958) said that the number of feelers in megalopa of eight species of Grapsidae ranges from three or four. There is only one feeler on the megalopa of *P. serrata.* Its tip is not serrated.

The megalopa of *P. serrata* can be distinguished from majid megalopae by the following:

1. In *P. serrata* there is a smooth feeler on the dactyl of periopod 5; none in majids.

2. There are no knobs, projections or modifications on the distal portion of the basal article of antenna 2 in *P. serrata.* There are more flagellar segments than the four that occur in majid megalopae.

3. The first segment of the peduncle of antenna 1 in *P. serrata* is inflated; its dorsal surface is flattened and concaved. This serves as a partial support of the eyestalk.

4. The gills are much better developed, having the elongated bud of the future podobranch and a bud of the epipodite on maxilliped 2. This is characteristic of the megalopae of multizoeal crabs (Yang, 1967).

The following characters distinguish the first crab stage of *P. serrata* from those of majid crabs:

1. In the first crab of *P. serrata*, the shape of the carapace is quite similar to that of the adult, as in the first crabs of some majids (*e.g.*, *Stenorhynchus* and *Epialtus*). However, the first crabs of other majids, *e.g.*, *Libinia, Microphrys* and *Macrocoeloma* are very different from the adult.

2. The inflated and laterally expanded first segment of the peduncle of antenna 1 now serves as the ventral support of the eyestalk. In the early postlarval stages of majid crabs (Acanthonychinae, Pisinae, and Mithracinae), the knob on the disto-dorsal surface of the basal article of antenna 2 locks into the ventral socket of the rostrum. Thus, the basal article is the ventral support (floor) of the eyestalk in the majid crabs.

3. The podobranch of maxilliped 2 is well developed in the first, and second and the parent adult crabs. As in adult *Cancer* (*cf.* Pearson, 1908), it is located along the anterior margin of the branchial series.

The developmental pattern, as well as the character of respective zoeal stages of the Parthenopinae, is completely different from those of the Majidae.

The subfamily Parthenopinae in the Parthenopidae is much larger group (about 128 species) than the other subfamily Eumedoninae (about 25 species). The latter occur in the Indo-Pacific and the species are mostly commensal (Balss, 1957). The larvae of the Eumedoninae are not yet known and may differ from those of the Parthenopinae.

The Hymenosomatidae, one of three families of Oxyrhyncha, has unique zoeal characters (lack of the dorsal and lateral spines, and peculiar telson) which differ from those of the Majidae and the Parthenopidae (Gurney, 1938). Boschi *et al.* (1969) found three zoeal stages and the carapace with only a rostral spine in the hymenosomatid, *Halicarcinus planatus* (Fabricius) reared from hatching. Gurney (1942, page 282) suggested that the atypical zoeal characters of Hymenosomatidae, provide strong evidence against the inclusion of the Hymenosomatidae in the Oxyrhyncha. Thus, it is now evident that in the Oxyrhyncha the zoeal characters of each family differ from those of the others, indicating that the Oxyrhyncha, like the Oxystomata, are a heterogeneous group.

In the adult classification, most revisers of Oxyrhyncha as mentioned in the previous section placed the Parthenopidae under Oxyrhyncha. Flipse (1930) also dealt with the Parthenopidae as Oxyrhyncha in his Siboga Expedition Report. However, Dana (1851, pages 426–427) considered the Parthenopidae as intermediate between the Maiinea and the cancroid crustacea because of the structure of the basal article of the antenna and the epistome, and Miers (1879, pages 635–636) stated that the Parthenopidae occupy a position almost intermediate between the rest of the Oxyrhyncha and certain Cancroidea in respect to the structure of the antennae. Miers (1879; page 641) also assumed the nearest affinities of the Parthenopidae in Oxyrhyncha are in the direction of *Inachus* through *Inachoides*. Cano (1893, page 580) provided a scheme, based on the adult characters, showing that the Parthenopidae diverged from the Inachidae-Majidae line.

On the other hand, quite a few workers have not placed the Parthenopidae under Oxyrhyncha. Most of the authors did not give reasons for their classifications. Strahl (1862a and b) separated the Parthenopidae from Oxyrhyncha and grouped it with Calappidae on the formation of the basal article of the antenna. The structure and formation of the rostrum, cephalothorax, antennae and orbits caused Ortmann (1893; pages 412–413) to place the parthenopid group under Cyclometopa. Guinot (1966 and 1967), seeking a "parthenoxystomienne" line, suggested the parthenopid crab (*Aethra*) and some oxystomous crabs (*Osachila, Hepatus, Hepatella* and *Actaeomorpha*) be united, because of their adult morphological characters.

Clearly, the systematic position of the Parthenopidae, based on adult characters, is still in question.

The larval characters of the Parthenopinae are completely different from those of Majidae and Hymenosomatidae. Parthenopid larvae are different at least from the Majidae in the number of zoeal stages, the formation and pattern of antenna 2, and the gill formation. The larvae of the subfamily Eumedoninae are completely unkown. However, the larval characters of the Parthenopinae (*Parthenope*) strongly suggest a relationship to the Brachyrhyncha rather than to the Oxyrhyncha.

The author is grateful to Dr. Anthony J. Provenzano, Jr. for his encouragement during the course of the present study. Mr. Henry B. Roberts of the United States National Museum confirmed the identification of the female. Dr. L. B. Holthuis and Dr. Raymond B. Manning criticized the manuscript. Mr. Robert Gore kindly helped in final revision of the manuscript. Mrs. C. Edith Marks helped with rearing of specimens.

SUMMARY

1. Larvae of an oxyrhynchous crab, *Parthenope* (*Platylambrus*) *serrata* (H. Milne Edwards), were successfully reared in the laboratory from hatching to the second crab stage on a diet of *Artemia* nauplii. Six zoeal stages, one megalopa and the first crab stage are described and illustrated.

2. Two series of larvae were reared at each of two temperatures, 20° and 25° C. The salinity ranged between 34 and 37‰. The mortality of the first zoeal stage reduced the initial population to less than half. The first crab stage was attained in approximately 30 days at 25° C, and in 45 days at 20° C, after hatching. The first zoeal stage and the megalopal stage showed more prolonged mean duration than intermediate stages and the pattern of mean duration was similar in both 20° and 25° C.

3. The major characteristics of four species of parthenopid first zoeae of known parentage are tabulated for comparison. The major distinctive characters of *Parthenope* larvae are presence of rostral, dorsal and lateral spines on the carapace, lunate telson with a dorsally located spine on each fork, normally six zoeal stages, and a smooth "feeler" at the tip of the fifth pereiopod in the megalopa.

4. The number of zoeal stages and the morphological characters are compared to those of oxyrhynchous crabs. The larval characters suggest that Parthenopinae should be removed from the superfamily Oxyrhyncha and placed in the Brachyrhyncha.

LITERATURE CITED

Aikawa, H., 1935. Inachidae ka oyobi kinen-shu no zoea youchū no keitai [The zoeal morphology of the family Inachidae and allied species]. *Zool. Mag. (Dobutsugaku Zasshi) Tokyo,* **47**: 217–227.

Aikawa, H., 1937. Further notes on brachyuran larvae. *Rec. Oceanogr. Wks. Japan,* **9**: 87–162.

Alcock, A. W., 1895. Materials for a carcinological fauna of India. No. 1. The Brachyura Oxyrhyncha. *J. Asiat. Soc. Beng.,* **64**: 157–291.

Balss, H., 1957. Abteilung Brachyura, Borradaile, 1907. Pages 1505–1672 in H. G. Bronns, Ed., *Klassen und Ordnungen des Tierreichs 5* (1) 7.

Boraschi, L., 1921. Osservazioni sulle larve dei Crostacei Decapodi, Brachiuri e Anomuri. *Memorie R. Comitato Talassografico Italiano,* **87**: 1–32.

Boschi, E. E., M. A. Scelzo and B. Goldstein, 1969. Desarrollo larval del cangrejo *Halicarcinus planatus* (Fabricius) en el laboratorio (Crustacea, Decapoda, Hymenosomidae), con observaciones sobre la distribución de la especie. *Bull. Mar. Sci.,* **19**: 225–242.

Bourdillon-Casanova, L., 1960. Le Meroplancton du Golfe de Marseille: Les larves de Crustacés Décapodes. *Rec. Trav. Sta. Mar. Endoume Fac. Sci. Marseille,* **30**(18): 1–286.

Cano, D. G., 1893. Sviluppo e Morfologia degli Oxyrhynchi. *Mitt. Zool. Sta. Neapel,* **10**: 527–583.

Costlow, J. D., Jr., 1965. Variability in larval stages of the blue crab, *Callinectes sapidus. Biol. Bull.,* **128**: 58–66.

Costlow, J. D., Jr., and C. G. Bookhout, 1959. The larval development of *Callinectes sapidus* Rathbun reared in the laboratory. *Biol. Bull.,* **116**: 373–396.

Dana, J. D., 1851. On the classification of the Maioid Crustacea or Oxyrhyncha. *Amer. J. Sci., Ser. 2,* **11**: 425–434.

Fagetti, G. E., 1960. Primer estadio larval de cuatro Crustaceos Braquiuros de la Bahia de Valparaiso. *Rev. Biol. Mar.,* **10**: 143–154.

Flipse, H. J., 1930. Die Decapoda Brachyura der Siboga-Expedition. VI. Oxyrhyncha: Parthenopidae. *Siboga Exped., Monog.,* **39**: 1–96.

Gamō, S., 1958. On the post larval stages of two species of crabs of the subfamily Varuninae, Grapsidae, Brachyuran Crustacea. *Zool. Mag. (Dobutsugaku Zasshi) Tokyo,* **67**: 373–379.

Garth, J. S., 1958. Brachyura of the Pacific coast of America. Oxyrhyncha. *Allan Hancock Pacif. Exped.,* **21**: 1–854.

GOURRET, P., 1884. Considérations sur la faune pélagique du Golfe de Marseille. *Ann. Mus. Hist. Natur. Marseille, Zool,* 2(2): 1–175.

GUINOT, D., 1966. Recherches préliminaires sur les groupements naturels chez les Crustacés Décapodes Brachyoures. I. Les affinités des genres *Aethra, Osachila, Hepatus, Hepatella* et *Actaeomorpha. Bull. Mus. Hist. Natur., Paris,* **38**: 744–762.

GUINOT, D., 1967. Recherches préliminaires sur les groupements naturels chez les Crustacés Décapodes Brachyoures. I. Les affinités des genres *Aethra, Osachila, Hepatus, Hepatella* et *Actaemorpha* (suite et fin). *Bull. Mus. Hist. Natur., Paris,* **38**: 828–845.

GURNEY, R., 1938. Notes on some decapod Crustacea from the Red Sea, VI: Some brachyuran larvae. *Proc. Zool. Soc. London,* **108**: 73–84.

GURNEY, R., 1942. *Larvae of Decapoda Crustacea.* Ray Society, London, 306 pp.

HEEGAARD, P., 1963. Decapod larvae from the Gulf of Napoli hatched in captivity. *Vidensk. Medd. Naturhist Foren. Kjobenhavn,* **125**: 449–493.

HOLTHUIS, L. B., 1959. The Crustacea Decapoda of Suriname (Dutch Guiana). *Zool. Verh.,* **44**: 1–296.

LEBOUR, M. V., 1928. The larval stages of the Plymouth Brachyura. *Proc. Zool. Soc. London,* **1928**: 473–560.

LEBOUR, M. V., 1944. Larval crabs from Bermuda. *Zoologica,* **29**: 113–128.

MIERS, E. J., 1870. On the classification of the maioid Crustacea or Oxyrhyncha, with a synopsis of the families, subfamilies, and genera. *J. Linn. Soc. Zool.,* **14**: 634–673.

MILNE EDWARDS, H., 1834. Histoire naturelle des Crustacés, comprenant l'anatomie, la physiologie et la classification de ces animaux. In *Librairie Encylopédique de Roret Volume 1,* Paris.

MIR, R. D., 1961. The external morphology of the first zoeal stages of the crabs, *Cancer magister* Dana, *Cancer antennarius* Stimpson and *Cancer anthonyi* Rathbun. *Calif. Fish Game,* **47**: 103–111.

ORTMANN, A., 1893. Die Decapoden-Krebse des Strassburger Museums. VII. Theil Abtheilung: Brachyura (Brachyura genuina Boas) II. Unterabtheilung: Cancroidea, 2. Section: Cancrinea, 1. Gruppe: Cyclometopa. *Zool. Jahrb. Abt. Sys. Oekol. Geogr. Tiere* **7**: 411–495.

PEARSON, J., 1908. Cancer. In *Liverpool Marine Biology Committee Memoirs XVI.* Williams and Norgate, London.

POOL, R. L., 1966. A description of laboratory-reared zoeae of *Cancer magister,* and megalopa taken under natural conditions (Decapoda Brachyura). *Crustaceana,* **11**: 83–97.

PROVENZANO, A. J., 1968. The complete larval development of the West Indian hermit crab, *Petrochirus diogenes* (L) reared in the laboratory. *Bull. Mar. Sci.,* **18**: 143–181.

RATHBUN, M. J., 1925. The spider crabs of America. *Bull. U. S. Nat. Mus.,* **129**: 1–693.

RICE, A. L., AND A. J. PROVENZANO, 1966. The larval development of the West Indian sponge crab *Dromidia antillensis* (Decapoda: Dromiidae). *J. Zool., London,* **149**: 297–319.

ROBERTSON, P. B., 1968. The complete larval development of the sand lobster *Scyllarus americanus* in the laboratory, with notes on larvae from plankton. *Bull. Mar. Sci.* **18**: 294–342.

SAKAI, T., 1938. *Studies on the crabs of Japan. III. Brachygnatha, Oxyrhyncha.* Yokendo Publishing Co., Tokyo.

STRAHL, C., 1862a. Über eine neue Species von *Acanthocyclus* Lucas, seine systematische Stellung und Allgemeines über das System der Dekapoden. *Monatsbericht Deutschen Akademie Wissenschaften Berlin,* (1861), **12**: 713–717.

STRAHL, C., 1862b. On a species of *Ruppellia,* Milne-Edwards, and the limit of the Brachyura. *Ann. Natur. Hist.,* **9**: 299–303.

WILLIAMS, A. B., 1965. Marine decapod Crustaceans of the Carolinas. *Fish. Bull. Fish. Wildlife Serv. U.S.,* **65**: 1–298.

YANG, W. T., 1967. A study of zoeal, megalopal, and early crab stages of some oxyrhynchous crabs (Crustacea: Decapoda). *Unpublished Doctoral dissertation, University of Miami,* 459 pp.

YATSUZUKA, K., 1957. Study of brachyuran zoea (artificial rearing and development). Pages 571–590 in *Suisan Gaku Shūsei* (Fisheries Compilation). Tokyo University Press, Tokyo.

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SEXUAL CYCLES AND MATURITY OF THE TURTLE, *CHRYSEMYS PICTA*

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The sexual cycles have been described for male *Sternotherus odoratus* (Risley, 1938), and both sexes of *Terrapene carolina* (Atland, 1951) and *Terrapene ornata* (Legler, 1960). Sexual cycles of the painted turtle, *Chrysemys picta* have been previously described for Nova Scotia females by Powell (1967) and for both sexes of Michigan *C. picta* by Gibbons (1968). A similar study was conducted on Pennsylvania *C. picta* which revealed certain differences in the spermatogenetic and oogenetic cycles and the attainment of sexual maturity as reported by Powell (1967) and Gibbons (1968). The results of this study are presented here.

METHODS

This study was conducted at the White Oak Bird Sanctuary, 3 miles north of Manheim, Lancaster County, Pennsylvania. Turtles were captured by hand, with a dip net, or in conventional hoop-net traps. Routine measurements of each turtle included the maximum plastron length, the straight line carapace length, the total shell width at the bridge, and the total shell height at the bridge. The third-claw length of both the fore and hind foot, the preanal, and the postanal tail lengths were also measured to determine the characters of sexual dimorphism (Table I).

Eighty adult specimens of *C. picta* (50 females, 30 males) were dissected and the reproductive tracts were removed to determine the male and female annual sexual cycles.

Fresh ovaries were examined from 30 females having plastron lengths ranging from 106.3 to 136.8 mm, dissected during the periods from 5 August to 31 October, 1966, and from 11 March to 30 July, 1967. Previous examinations (August, 1965) of reproductive tracts of 20 other females had indicated that specimens of 110 mm plastron length were sexually mature. All ovaries were weighed before preservation. Follicles, *corpora lutea,* and *corpora albicantia* were counted and measured after preservation. Follicles less than one millimeter in diameter were not counted. Follicles and oviducal eggs were recorded separate for the right and

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left sides. *Corpora lutea* and *albicantia* were studied under a binocular dissecting microscope. No histological studies were made of the female reproductive tract.

Fresh testes were examined from 30 males having plastron lengths ranging from 87.8 to 112.9 mm, dissected during the periods from 23 August to 26 September, 1966 and 5 March to 13 August, 1967. All testes were weighed, and the greater diameter was measured before preservation. The testes were fixed in 10 per cent neutral formalin and after two weeks transferred to 80 per cent ethyl alcohol for storage. Later the testes were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

All measurements in the field and laboratory were made to the nearest tenth of a millimeter with dial calipers. A triple-beam balance was used to determine all weights.

TABLE 1

A summary of sexual dimorphism in Chrysemys picta

Character	Range	Male mean	S.D.	Range	Female mean	S.D.
Plastron length	70.9–119.2	99.6	10.1	80.8–142.1	120.9	14.2
Shell width	67.0–99.5	81.0	6.6	78.5–114.3	94.4	8.6
Shell height	28.7–49.7	38.2	3.5	31.9–59.5	48.5	6.3
Third forefoot claw	5.1–13.0	10.8	1.4	3.7–9.2	6.6	0.7
Forefoot claw/hind foot claw	2.1–3.2	2.7	0.4	0.9–1.6	1.3	0.3
Preanal tail length	5.7–20.0	13.1	3.2	0.5–14.8	6.8	2.6
Postanal tail length	16.0–33.7	27.3	2.9	21.7–41.0	31.0	4.5
Total tail length	28.9–51.8	40.1	3.6	26.2–45.7	36.9	3.3
Tail length/plastron length	0.38–0.48	0.42	0.03	0.25–0.31	0.28	0.02

RESULTS

Female sexual cycle and maturity

Ovaries weighed the most during April and May (Fig. 1). This corresponds to the mating season and many large ovocytes were found on the ovaries at this time. The ovaries weighed the least during July, August, and September, or after ovulation and nesting has occurred. No correlation was found between plastron length and ovarian weight, as smaller females often had heavier ovaries on the same date as larger females. For example, a female with a plastron length of 107.6 mm sacrificed on 23 August, 1966, had an ovarian weight of 12.6 grams while another female, 136.8 mm in length, sacrificed the same day, had an ovarian weight of only 9.4 grams. Enlarged ovocytes (15 to 25 mm) were present throughout the study periods, but were most numerous during April and May and least numerous during July and August. Cagle (1954) reported that female specimens of *C. picta* collected during May, June, and early July and containing oviducal eggs usually had large ovocytes still present. He assumed that the ovocytes represented second and third broods that would be deposited that same season. Although careful searches were made from April through September, no evidence was found at White Oak to support Cagle. Fresh nests and nesting females were only found during June. Oviducal eggs were only found during late May, June, and early July. The oviducal eggs examined were pinkish white and slightly translucent, but became white shortly after their removal. They ranged in length from 23.1 to 33.0 mm (mean 28.4) in diameter from 15.4 to 20.1 mm (mean 17.5) and in weight from 5.3 to 6.8 g (mean 5.9).

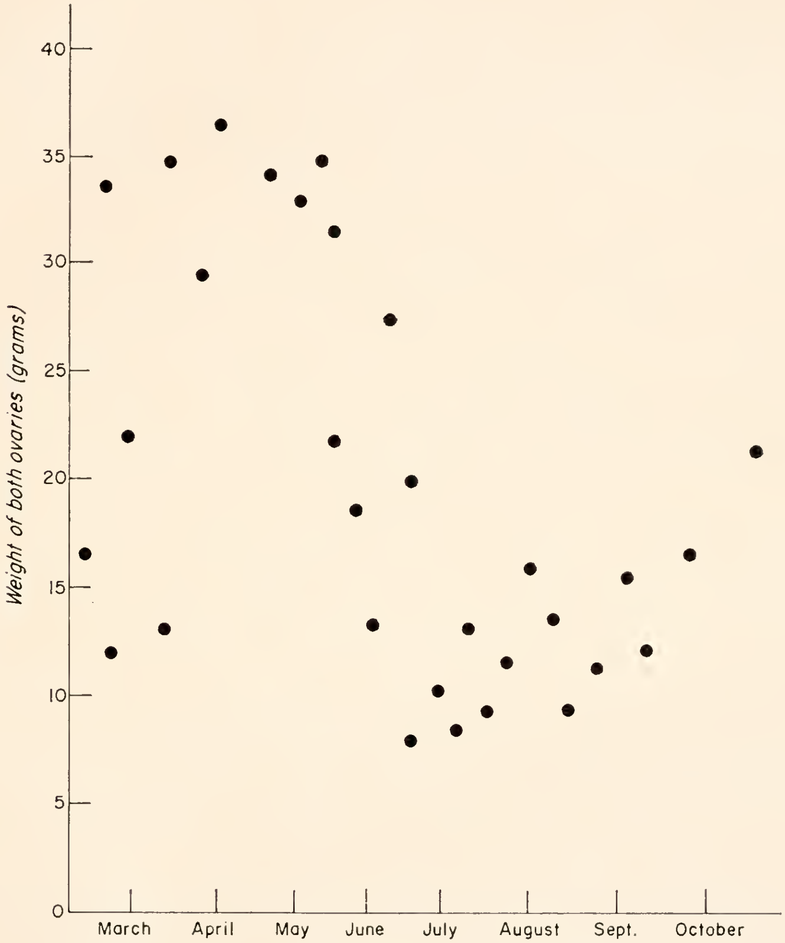

FIGURE 1. Seasonal fluctuations in ovarian weight in thirty specimens of *Chrysemys picta.*

The follicles found on the ovaries could be grouped by diameter as: large (greater than 15 mm), medium (11 to 15 mm), and small (6 to 10 mm) after the system proposed by Legler (1960). Those smaller than 6 mm were not considered. Mature follicles had diameters of 20 to 25 mm. Both Powell (1967) and Gibbons (1968) reported 18 mm as the maximum size of the follicles they measured. (Cagle (1944) considered female specimens of *Chrysemys scripta* to be mature if they contained follicles of 15 mm or greater. All female specimens of

C. picta with the largest follicle 10 mm or more in diameter had two or more additional groups of follicles present on the ovaries (6 to 9 mm in diameter and less than 5 mm). Apparently, these groups represent follicles which would mature and be ovulated during successive mating seasons. None were found to reach mature size before October, and therefore, second nestings as proposed by Cagle (1954), Powell (1967), and Gibbons (1968) would not occur in southeastern Pennsylvania.

The ovarian cycle begins in July and August after ovulation and nesting have occurred. Many small follicles form on the germinal ridges of the ovaries. The ovocytes within the follicles increase in size and by late September and October there is little difference in size between these and the June size of the ovocytes that were not ovulated (about 15 mm). Those not ovulated in June grow and reach mature size by early October (about 20 mm). This differs from Gibbons' (1968) and Powell's (1967) findings of little change in size during the summer. There is little change in size during the winter. Atland (1951) suggested that *Terrapene carolina* follicles grow to nearly mature size in the season preceding ovulation and remain quiescent over winter. This appears also to be the case in *C. picta*. Atland also thought that some of the enlarged follicles were absorbed during hibernation. The follicles of White Oak *C. picta* were of mature size in March and remained that way until the ovulation period (May and June). This differs from what Gibbons (1968) found in his Michigan *C. picta*. He reported that specimens taken in March contained yolked follicles 15 to 16 mm in diameter. These remained this size until May when they grew to mature size (about 18 mm).

TABLE II

Ovarian activity in ten specimens of Chrysemys picta

Left ovary		Right ovary	
Corpora lutea	Follicles (diameter 15+ mm)	*Corpora lutea*	Follicles (diameter 15+ mm)
4	2	0	4
3	1	1	3
2	5	4	0
2	2	3	1
3	1	1	2
2	3	4	2
5	1	0	3
1	2	5	0
4	2	1	3
4	2	2	3

Ovarian activity alternates in *C. picta*. Counts of *corpora lutea* showed one ovary more active than the other in a given season, and higher counts of enlarged follicles (15 mm in diameter or larger) indicated that the opposite ovary would be more active in the following season (Table II). Legler (1960) reported this same condition in *Terrapene ornata*. The *corpora lutea* are cuplike in shape and 7.5 to 9.0 mm in diameter. The number of oviducal eggs always equalled the number of *corpora lutea*. Involution of the *corpora lutea* occurs very rapidly and after a month they are barely visible on the ovarian surface and can be referred to as *corpora albicantia*. The *corpora albicantia* disappear when a new clutch of eggs is ovulated.

Extrauterine migration of ova has been reported in *C. picta*, *C. scripta*, *T. ornata*, and *Emydoidea blandingi*, by Legler (1958) and in *Sternotherus odoratus* by Tinkle (1959). An examination of the female painted turtles from which oviducal eggs were removed revealed no evidence of ovular migration. All had equal numbers of *corpora lutea* and oviducal eggs on the same side. Legler (1960) thought such migration may serve to redistribute eggs to the oviducts when the ovaries are functioning at unequal rates.

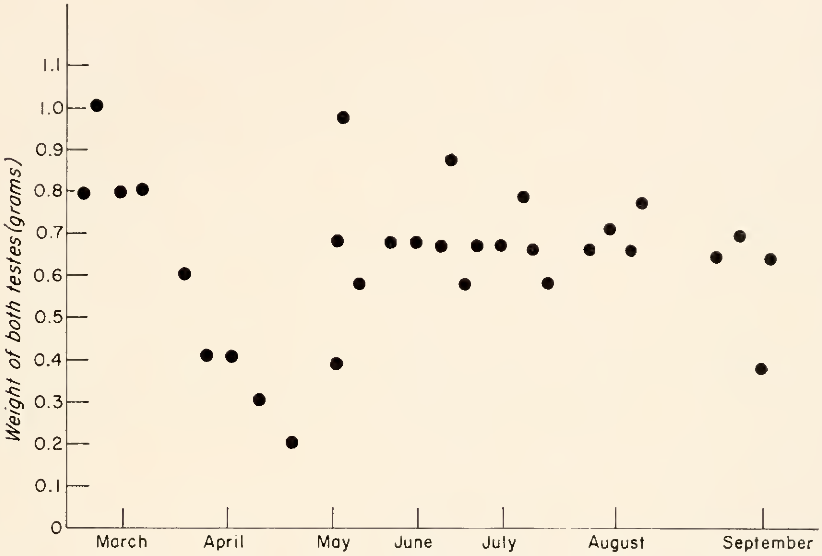

FIGURE 2. Seasonal fluctuations in testes weight in thirty specimens of *Chrysemys picta*.

Females were considered sexually mature if they: 1) contained follicles with diameters greater than 15 mm; 2) contained oviducal eggs; 3) were found mating; or 4) were found nesting. Only 7 females of 16 examined with plastron lengths below 100 mm contained follicles of 15 mm. The smallest had a plastron of 80.8 mm. Four had plastron lengths between 90 and 99 mm. All 7 showed 5 years growth annuli. All females examined over 100 mm plastron length contained some follicles of 15 mm. All of the females found containing oviducal eggs, mating, or nesting, were over 110 mm in plastron length and at least 5 years old. White Oak females were mature at 110 mm plastron length after their fifth year. Possibly some mature at a smaller size (between 100 and 110 mm). Cagle (1954) reported 20 Illinois females containing eggs were 126 to 160 m min plastron length, 14 Tennessee females 106 to 138 mm, and a single Michigan female was 152 mm. He assumed that a female having ovarian follicles 10 mm or greater in diameter was capable of depositing eggs; and found 41 Illinois females, plastron lengths

122 to 162 mm, and a single Louisiana female, length 125 mm, with follicles of this size. He stated that females became sexually mature when they reach a plastron length of 120 to 130 mm. Gibbons (1968) reported that sexual maturity in southwestern Michigan was attained when females reached a plastron length of about 110 to 120 mm (at about 10 years of age).

Male sexual cycle and maturity

Testes weighed the most during March following emergence from hibernation (Fig. 2). The testes contained much sperm at this time and additional weight was possibly due to the proliferation of the sustentacular cytoplasm. Weight decreased steadily during April and May as the sperm passed out of the testes, and into the epididymides. Weight increased in June as sperm maturation again began. The greatest diameter varied little, ranging from 6 to 8 mm during each month. Gibbons (1968) reported changes in the size of the testes. He reported the testes to be reduced in size from March to June, enlarged from July to September and to be small again in October. The changes in weight agree with those reported by Gibbons (1968) for Michigan *C. picta*.

Spermatogenesis began in March when a few spermatogonia first appeared. At this time there were many Sertoli cells present and some cellular detritus was contained in the lumen of the seminiferous tubules. Sperm produced in the previous cycle were also present in the lumen. As March progressed the clear cytoplasm of the cells extended into the lumen of the turtle.

During April there was much detritus in the lumen, and the sperm started to pass out of the tubules and into the epididymides. The cells bordering the tubule were no longer clear or extending into it. There were about an equal number of Sertoli cells as primary spermatocytes. As the month passed, a second layer of spermatocytes was formed.

In May there were two or three rows of spermatocytes, both primary and secondary, surrounding the Sertoli cells which were decreasing in number. Spermatids were now present in small numbers. Detritus and some sperm still remained in the lumen.

A few new sperm appeared around the border of the lumen in early June. Many of the lumens were now clear of detritus. Spermatids increased in numbers. Spermatocytes were present, but the Sertoli cells had practically disappeared. In late June many newly formed clumps of sperm could be seen around the lumen border.

July and August were the most active periods of sperm production. By late July large numbers of sperm were found clumped in the lumens and by the end of August filled most of the lumen and lined the borders.

During September the sperm moved to the center of the lumen so that few remained at the border at the end of the month. The cycle appeared complete by this time. Gibbons (1968) reported that the sperm passed into the epididymides during October. Figure 3 shows stages of the cycle.

Risley (1938) found the testes of *Sternotherus odoratus* to be largest in August and smallest in early May. Recession of testes in spring coincided with the mating period and later increases in size with increasing spermatogenic activity

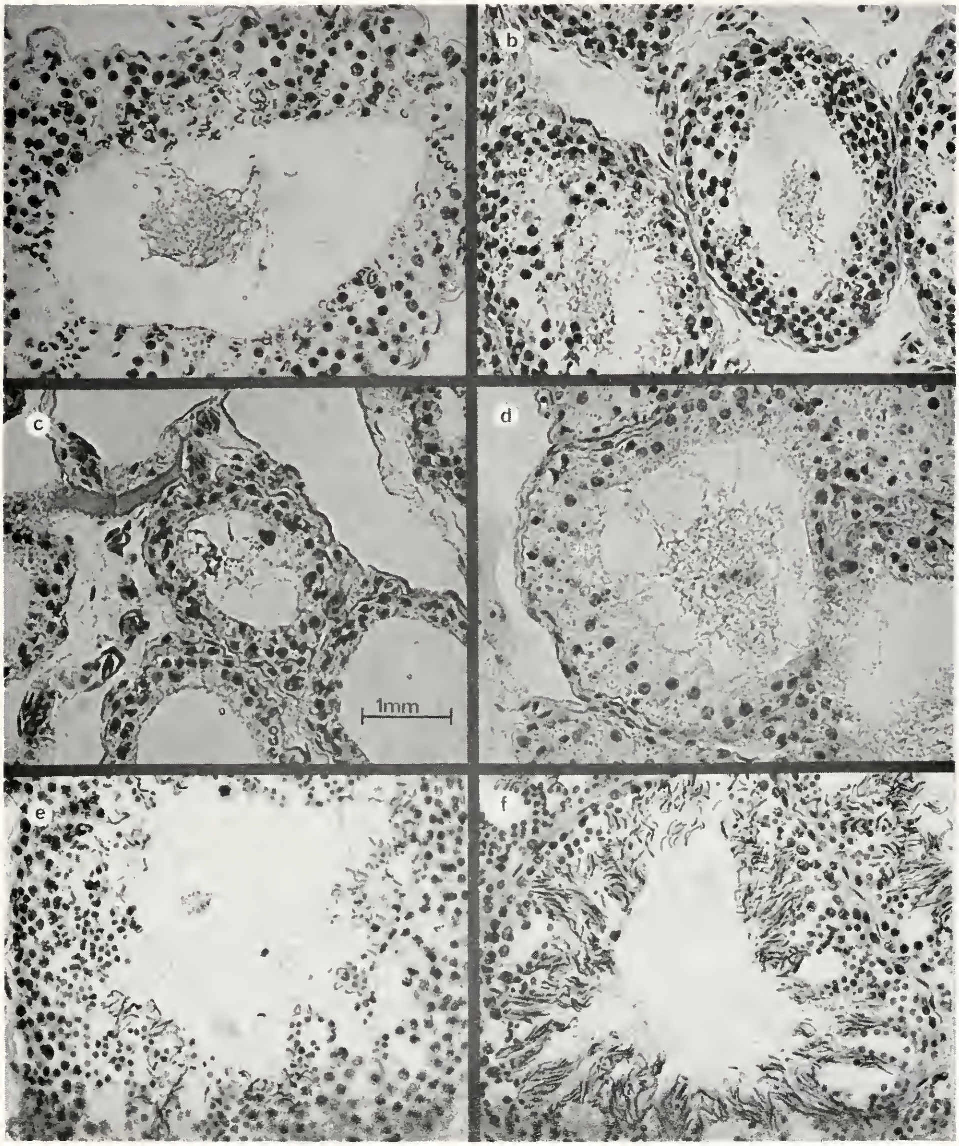

FIGURE 3. Representative stages in the spermatogenic cycle of *Chrysemys picta*. Letters a to f, respectively, are sections of testes obtained on 14 March, 4 April, 11 May, 12 June, 12 July, and 23 August.

and enlargement of the seminiferous tubules. Atland (1951) reported that the cycle of *Terrapene carolina* was essentially like that of *S. odoratus*. Legler (1960) showed the cycle of *Terrapene ornata* began in early May and ended in late October and except for a longer length was the same as that of *S. odoratus* and *T. carolina*. The spermatogenic cycle of *C. picta* beginning in March and ending in September is even longer, but in main points does not differ from those species previously mentioned.

Male specimens of *C. picta* were considered sexually mature if they contained mature sperm in their testes or epididymides. The smallest turtle containing mature sperm had a plastron length of 87.8 mm and showed 5 growth annuli. This turtle was sacrificed on 19 September, 1966, and possibly had mated the previous spring. Mature males have elongated forefoot claws (over twice as long as those of the hind feet), and elongated preanal tail lengths (Table I). Males first showed these characters at 70 mm plastron length in their fourth year. Apparently, males mature during their fourth year in Pennsylvania, but do not mate until the spring of their fifth year. Gibbons (1968) reported that male *C. picta* in southwestern Michigan mature when they reach 80 mm plastron length and showed some to be mature in their fourth year. Cagle (1954) reported male *C. picta* reached maturity at 90 mm in Michigan, 70 mm in Illinois, and reported Louisiana males sexually mature at 55 and 62 mm, but other males in the size range 50–60 mm were sexually inactive. Cagle (1954) stated that males of the southern populations apparently may become sexually mature during the first year of life (in one complete growing season), and the northern males require at least two and possibly three seasons to attain maturity. Cagle (1948) reported male *Chrysemys scripta* normally become mature at plastron lengths of 90 to 100 mm but occasionally individuals may become mature at a smaller size. *Chrysemys scripta* in their first mature season may be 2 to 5 years old. The attainment of maturity in the genus *Chrysemys* is apparently a factor of size rather than age.

Discussion

The variations in the sexual cycles and attainment of maturity between the specimens of *Chrysemys picta* in this study and those studied by Cagle (1954), Powell (1967), and Gibbons (1968) are of interest.

Climatic conditions greatly influence the physiological activity of turtles. Cold temperatures reduce the metabolic rate of *Chrysemys picta* (Rapatz and Musacchia, 1957) and probably also reduce the activity of the reproductive organs. White Oak painted turtles were active in all months except February (Ernst, 1969), and although Gibbons' (1968) females had similar ovulatory periods to those from White Oak, the annual activity period in southwestern Michigan was shorter (Gibbons, 1967). Sexton (1959) also reported a shorter annual activity period during his study of *C. picta* in southern Michigan. Although no figures are available on the length of the annual activity period in Nova Scotia, it is probably also shorter than that in southeastern Pennsylvania. This is caused by the earlier onset of colder water temperatures and the delayed thawing of the ice cover in spring.

According to Goode (1953) both southern Michigan and Nova Scotia have a continental forest climate with cool summers while southeastern Pennsylvania has a continental forest climate with warm summers. Nova Scotia has a surface temperature below 32° F in winter and from 50° to 68° F in summer. Both southern Michigan and southeastern Pennsylvania have surface temperatures below 32° F in winter and above 68° F in summer. During January the normal temperatures of both southern Michigan and Nova Scotia range between 20° and 30° F, while those of southeastern Pennsylvania range between 30° and 40° F. The normal July temperatures of southern Michigan and southeastern Pennsylvania range between 70° and 90° F while those of Nova Scotia only range between 50° and 70° F.

A shorter period of development caused by prolonged colder water temperatures could explain why the ovocytes of females from the more northern populations do not grow as large as do those from White Oak females. It would be interesting to compare data from Louisiana females. This could also explain the earlier maturity of both sexes at White Oak. Cagle (1954) has reported an inverse relationship between the attainment of sexual maturity and latitude in *Chrysemys picta,* as shown in the present study. Tinkle (1961) reported that in *Sternotherus odoratus* both southern males and females reach sexual maturity at a smaller size than do the northern sexes.

Inhibition by cooler temperatures may explain the lack of growth in ovocytes in Nova Scotia during the summer, but it is not clear why such a difference should exist between the Michigan and Pennsylvania populations which both have warm summers.

Painted turtles from more southern localities are known to have longer reproductive periods. Cagle (1954) defined the nesting season of *Chrysemys picta* as that period in which females may contain oviducal eggs and reported a nesting season of early April to the last of July in Louisiana and from 12 May to 22 July in Illinois. Presumably this difference is caused by the warmer climate in Louisiana.

Multiple nestings as supposed by Cagle (1954), Powell (1967), and Gibbons (1968) have never been proven. Since no female *Chrysemys picta* has been observed nesting more than once in a given season, this difference may not exist. If multiple nestings do occur they would be restricted to southern populations because of the short northern egg-laying season.

The difference in the sexual cycles and attainment of maturity shown in this study point out that too often the results of a study of one population of a species are fallaciously taken for granted as being true for all such populations throughout the species' range. Gibbons and Tinkle (1969) have pointed out similar variations in the clutch size of *C. picta* populations in a single geographic area. There is a critical need for more information on the factors affecting reproduction in turtle populations.

I wish to thank J. Robert Heckman for preparing the histological sections of *Chrysemys testes,* Geoffrey Kampe for taking the photomicrographs, and Dr. Roger W. Barbour for his advice and criticisms.

This paper is a portion of a dissertation presented to the faculty of the Department of Zoology, University of Kentucky, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Summary

1. A mature male *Chrysemys picta* can be distinguished from a female by its long forefoot claws, thickened base of the tail, and short post-anal tail length.

2. Males become sexually mature when they reach a plastron length of 80 to 90 mm. They usually mature in their fourth year, but probably do not mate until the spring of the fifth year.

3. All sexually mature females contain follicles with diameters greater than 15 mm. All females containing oviducal eggs found mating or nesting were over 110 mm in plastron length and at least five years old.

4. Sexual maturity is apparently a factor of size rather than age.

5. Spermatogenesis begins in March with the most active period of sperm production occurring in July and August. The cycle is completed in September when the sperm start to pass into the epididymis where they are stored during the winter.

6. The ovarian cycle in southeastern Pennsylvania begins in July and August after ovulation and nesting, and is completed by the end of October. No change occurs during the winter. This differs slightly from the cycle of more northern females.

7. The ovarian activity alternates, and extrauterine migration of ova is known to occur.

LITERATURE CITED

Atland, P. D., 1951. Observations on the structure of the reproductive organs of the box turtle. *J. Morphol.*, **89**: 599–621.

Cagle, F. R., 1944. Sexual maturity in the female of the turtle *Pseudemys scripta elegans. Copeia*, **1944**: 149–152.

Cagle, F. R., 1948. Sexual maturity in the male turtle, *Pseudomys scripta troostii. Copeia*, **1948**: 108–111.

Cagle, F. R., 1954. Observations on the life cycles of painted turtles (genus *Chrysemys*). *Amer. Midland Natur.*, **52**: 225–235.

Ernst, C. H., 1969. Natural history and ecology of the painted turtle, *Chrysemys picta* (Schneider). *Ph.D. dissertation, University of Kentucky*, **207** pp.

Gibbons, J. W., 1967. Population dynamics and ecology of the painted turtle, *Chrysemys picta. Ph.D. dissertation, Michigan State University*, 111 pp.

Gibbons, J. W., 1968. Reproductive potential, activity, and cycles in the painted turtle, *Chrysemys picta. Ecology*, **49**: 399–409.

Gibbons, J. W., and D. W. Tinkle, 1969. Reproductive variation between turtle populations in a single geographic area. *Ecology*, **50**: 340–341.

Goode, J. P., 1953. *Goode's World Atlas.* Rand McNally and Co., New York, 272 pp.

Legler, J. M., 1958. Extra-uterine migration of ova in turtles. *Herpetologica*, **14**: 49–52.

Legler, J. M., 1960. Natural history of the ornate box turtle, *Terrapene ornata ornata* Agassiz. *Univ. Kans. Publ. Mus. Natur. Hist.*, **11**: 527–669.

Powell, C. B., 1967. Female sexual cycles of *Chrysemys picta* and *Clemmys insculpta* in Nova Scotia. *Can. Field Natur.*, **81**: 134–140.

Rapatz, G. L., and X. J. Musacchia, 1957. Metabolism of *Chrysemys picta* during fasting and during cold torpor. *Amer. J. Physiol.*, **188**: 456–460.

Risley, P. L., 1938. Seasonal changes in the testes of the musk turtle, *Sternotherus odoratus. J. Morphol.*, **63**: 301–317.

Sexton, O. J., 1959. Spatial and temporal movements of a population of the painted turtle, *Chrysemys picta marginata* (Agassiz). *Ecol. Monogr.*, **29**: 113–140.

Tinkle, D. W., 1959. Additional remarks on extra-uterine migration of ova in turtles. *Herpetologica*, **15**: 161–162.

Tinkle, D. W., 1961. Geographic variation in reproduction, size, sex ratio and maturity of *Sternothaerus odoratus* (Testudinata: Chelydridae). *Ecology*, **42**: 68–76.

Reference: *Biol. Bull.*, **140**: 201–214. (April, 1971)

FEMALE SEXUAL BEHAVIOR AS THE MECHANISM RENDERING *AEDES AEGYPTI* REFRACTORY TO INSEMINATION [1]

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The yellow fever mosquito, *Aedes aegypti* (L.), is one of the most thoroughly studied insects from the standpoint of both basic and applied science. Because of its importance as a vector of human diseases, a great deal of literature has accumulated concerning aspects of its systematics, distribution, bionomics, disease relationships and control. In spite of this intensive study, little was known until recently about its mating behavior. Recent research on sexual behavior of mosquitoes has indicated that many widely accepted concepts were incorrect. Moreover, several points of controversy remain.

One of the first serious investigations of sexual behavior in *A. aegypti* before and during coitus was undertaken by Roth (1948). In this paper he firmly established that flight sound of the female mosquito attracted males and initiated copulatory behavior. Later, Spielman (1964) and Jones and Wheeler (1965) described the act of copulation in detail and attempted to explain the mechanism by which semen is transferred by the male to the female.

Early students of the behavior of *A. aegypti* observed that females begin to copulate soon after emergence and continue to do so throughout life. Most assumed that the terms "mating," "copulation" and "insemination" were synonymous, thus concluding that most copulations result in insemination. MacGregor (1915) stated that copulation takes place as soon as females are ready to fly. Roth (1948) noted that most females are mated within 105 to 145 minutes after emergence, and recorded a single female that underwent 50 successful copulations by 11 different males during a one hour period. Finally, Gillett (1955) observed that, in captivity, a single female of *A. aegypti* may mate as many as 40 times before her first blood meal.

There has been considerable confusion and improper usage of terms affecting mosquito reproduction. The following is a partial list of terms related to behavior and reproduction as used herein:

Mating = That portion of sexual reproduction which begins with the search for a sexual partner and ends with successful insemination. Copulation or coitus represents only one portion of mating. Mating behavior includes swarming and other mate-finding mechanisms.

Copulation = The act of sexual intercourse. Coitus, coition, sexual union. In mosquitoes, copulation is a rather mechanical process. It requires the bodies of

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both partners to be in the correct position and their genitalia to be at the proper degree of connection so that ejaculation and insemination can take place.
Coupling = The events occurring when a sexually aroused male seizes a female and establishes genital contact. The male remains in contact and tries to achieve a position of coitus that will permit insemination. He may strive for the copulatory position but not achieve it. A male encountering a sexually refractory female may couple, but cannot copulate.
Insemination = The steps that occur between male ejaculation and deposition of sperm in the spermathecae. Sperm and seminal fluid are introduced into the bursa copulatrix of the female and, within a few minutes, sperm begin to migrate to the spermathecae where they are stored until the time of fertilization.

A major revision in our understanding of the sexual behavior of *A. aegypti* occurred when it was demonstrated that females of this species are monogamous (Craig, 1967; Spielman, Leahy and Skaff, 1967). Copulation by a mature virgin female results in insemination. Although the female appears to copulate with other males, she is refractory to a second insemination for life. Subsequent insemination is prevented through the action of a substance from the male accessory glands that is transferred to the female in the seminal fluid. This substance has been designated "matrone" (Fuchs, Craig and Hiss, 1968).

Virgin females can be rendered refractory to insemination by implants of accessory glands from adult males (Craig, 1967; Spielman *et al.*, 1967), by injection of an aqueous extract of glands (Craig, 1967), or by injection of material extracted from lyophilized whole bodies of males (Fuchs *et al.*, 1968). The partially purified substance, matrone, has been shown to be a protein (Fuchs, Craig and Despommier, 1969).

A second major revision of the life history of *A. aegypti* came with the description of a post-emergence refractory period (Gwadz and Craig, 1968; Lea, 1968). During this refractory period, which may last 36 to 60 hours, females may couple repeatedly, but they are not inseminated. Lea (1968) showed that the onset of sexual receptivity could be prevented by removal of the corpora allata and restored by gland implants. He concluded that sexual receptivity was influenced by juvenile hormone, a product of the corpora allata. Later, Gwadz, Lounibos and Craig (1971) demonstrated that the post-emergence refractory period could be reduced by more than 70% by the topical application of a synthetic analogue of juvenile hormone to pupae or teneral adults.

The mechanisms which permit a female of *A. aegypti* to couple without being inseminated have become a matter of controversy. Both young virgin females and previously inseminated females appear to copulate repeatedly but they are not inseminated. Craig (1967) felt that matrone might stimulate the inseminated female to hold her vaginal lips closed, thereby preventing the introduction of semen. Gwadz and Craig (1968) hypothesized that young virgin females are not inseminated because they withdraw their terminalia and prevent males from completing firm genital union.

On the other hand, Spielman *et al.* (1967, 1969) concluded that the explanations offered by Gwadz and Craig served only to describe some of the behavioral characteristics of females refractory to insemination. Spielman and co-workers felt that these behavioral traits alone were not major barriers to insemination. It was their hypothesis that the failure of effective insemination was due in part to the physical condition of the bursa copulatrix of the female at the time of copulation. They attempted to show that young or previously inseminated females are unable to alter the consistency of the semen as it is introduced into the bursa. They felt that the seminal mass is expelled by the female or withdrawn by the male at the termination of coitus. They further contended that sperm and seminal fluid is transferred to females of all ages and sexual experience; however, a female is capable of retaining semen from only one copulation during her lifetime.

Observation on insemination capacity of males are relevant to this controversy. A male can inseminate 5 to 7 females but has little or no capability for sperm replenishment once he is depleted (Gwadz and Craig, 1970; Hauserman and Nijhout, in press). If, as Spielman contends, copulations with refractory females involve semen transfer and subsequent semen loss, this loss should be reflected as a reduction in the capacity of a male for further insemination. The experiment of Powell (Craig, 1967) shows that male capacity for insemination is not reduced after exposure to previously inseminated females. The present study was undertaken to amplify the preliminary results of Powell. Moreover, an attempt was made to analyze the nature of post-emergence and post-insemination refractory responses.

MATERIALS AND METHODS

All experiments were conducted with the ROCK strain of *A. aegypti*. ROCK is a relatively large, uniform, vigorous strain used in a number of laboratories and may be considered as characteristic of the type form of *A. aegypti aegypti*. This strain was originally obtained from D. W. Jenkins in 1959 and is the most commonly used strain at the Vector Biology Laboratory of the University of Notre Dame.

All rearing was conducted in accordance with the methods of Craig and VandeHey (1962). Larvae were reared in enamel pans in 2500 ml of tap water at a density of approximately 200 larvae per pan. Larvae were fed a suspension of liver powder (Nutritional Biochemicals Corporation, Cleveland, Ohio) at a concentration of 10 g powder per 1000 ml of tap water. The feeding schedule consisted of 10 ml of suspension on the day of egg hatching (day 0), 10 ml on day 1, and 20 ml each on days, 2, 3 and 4. Pupation began late on day 4 and was completed by day 5.

Care was taken to insure uniform growth and pupation, and pans were discarded if the larvae they contained failed to achieve greater than 95% pupation by the middle of day 5.

Sexes were separated by size and transferred to petri dishes (15 cm diameter) lined with moist paper towelling. These dishes were placed in gallon cardboard ice cream containers covered with bolting cloth. Adult emergence took place in these containers.

All rearing and experimentation was performed at 27 ± 1° C and 80 ± 5% relative humidity. Day length was 16 hours.

In experiments where the age of the female was of importance, emerging females were collected from the cages at hourly intervals. All females used in these age-related studies were of known age ±30 minutes. The ages of females used in all other studies were known with ±12 hours. Newly emerged adults were transferred to gallon containers and maintained on apple slices as a source of food.

In experiments where previously inseminated females were required, 2–3 day old females were placed with a surplus of males for two days. This treatment resulted in approximately 95% insemination of females.

Determination of sexual receptivity

Females of *A. aegypti* do not go through any obvious precopulatory courtship behavior. Therefore, the criterion for sexual receptivity used herein was insemination of the female upon exposure to males. Copulation and insemination of receptive virgin females normally takes place within minutes of exposure; indeed, the entire act can be completed in less than 10 seconds. To determine if females are receptive or refractory to insemination, the following procedures were utilized. A group of 10 to 15 females was placed in a gallon cage containing 30 to 40 males of mixed ages from 2 to 10 days old. Exposure time was 24 hours. Females were then dissected in saline and their bursae and spermathecae examined for sperm with a compound microscope at 100 ×. Scoring for insemination was on the basis of presence or absence of sperm in the spermathecae. In a second method for determination of receptivity, individual females were exposed to 1 or 2 males in a modified lantern chimney 10 × 12 cm, instead of a gallon cage. The chimneys were rotated on their sides to induce mosquito flight until couplings occurred. Chimney rotation was stopped after a mosquito pair coupled and the duration of genital contact was recorded. The female was then removed and examined for insemination.

An apparatus was designed to allow observation of the behavioral responses of female mosquitoes to mating attempts by males. Individual females were lightly etherized and glued by the mesonotum to the head of a pin with a fast-drying plastic cement (Dekadhese, Donald Tullock, Jr., Chadds Ford, Pennsylvania). After recovery from anaesthesia, the female was lowered into the viewing cage. Flight was stimulated by directing a stream of air over the female; copulation attempts by the free-flying males quickly ensued. Observation was simplified by viewing through a stereoscope mounted horizontally in front of the cage. The stereoscope had a magnification potential of from 10 to 70 × and was fitted with a photographic attachment.

Matrone preparation and assay

An extract of the active principle of male accessory glands, matrone, was prepared in accordance with the method described by Craig (1967) with minor modifications. Accessory glands were obtained from virgin males, 4 to 6 days old, by pulling the terminalia of these males while holding the thorax. One hundred tails in 1 ml of *A. aegypti* saline (Hayes, 1953) were sonicated for 10 seconds and subjected to $-5°$ C centrifugation at 18,000 *g* for 20 minutes. The residue was discarded and the supernatant frozen until needed.

Assay procedures to determine activity consisted of: (1) intrathoracic injection of 20 virgin females with 1 μl of solution, (2) 24 hour recovery period followed by a 24 hour exposure to a surplus of males, (3) examination of females for evidence of insemination. Active matrone renders more than 95% of the injected females refractory to insemination for life. Saline-injected control females show greater than 90% insemination after a 24 hour exposure to males.

Method for isotopic labelling

Third instar larvae (72 hours old) were transferred into a paper pint rearing cup containing 200 ml of water. An aqueous solution containing C^{14} was added to give a concentration of 0.05, or 0.10 microcuries per ml. The isotope used was either L-isoleucine-C^{14} or yeast protein (whole)-C^{14}, obtained respectively from New England Nuclear or the International Chemical and Nuclear Corp. The regular larval feeding regime was continued; liver powder suspension was added to all containers. Each cup contained fifty larvae. The larvae remained in the labelled solution until pupation, generally a period of three days. Pupae were sexed according to size (males half as large as females) and transferred to emergence containers.

TABLE I

Radioactivity measurements of adult male mosquitoes from larvae reared in labelled solution from third instar to pupation

Treatment*	Conc. μc/ml	Adult males			
		No. tested	Counts/minute		
			Minimum	Maximum	Mean
Isoleucine-C^{14}	0.05	10	14,610	62,277	27,681
Yeast protein-C^{14}	0.10	10	6,134	16,599	13,462

* 50 larvae, 72 hours old, placed in 200 ml water containing liver powder and isotope; larvae remained in this solution for three days, until pupation.

Assays of radioactivity were obtained using a Packard Tri-Carb liquid scintillation spectrometer (model 33651). The adult mosquitoes to be tested were crushed individually on a small piece of filter paper and were placed in 20 ml glass counting vials with foil-lined screw caps. The scintillation fluid was composed of 7 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP (p-Bis 2-(5-phenyloxazolyl)-benzene) per liter of toluene. Data are reported in counts per minute; the background count averaged 25 counts/minute.

Radioactivity of labelled males is indicated in Table I. Isoleucine-C^{14} at 0.05 μc/ml gave males averaging 27,681 cpm, whereas yeast protein-C^{14} gave males with an average of 13,462 cpm. Perhaps the labelled amino acid was assimilated more rapidly, thus explaining the higher mean count from the less concentrated solution.

RESULTS

Sexual receptivity and sperm transfer

A single virgin male was exposed to 10 receptive virgin females in a gallon-sized cage for a 24 hour period, then placed with 10 additional females for a second 24 hours. After each exposure the females were dissected and examined for evidence of insemination. During the first 24 hour period, the 33 males tested inseminated an average of 5.70 females per male (Table II). However, during the second 24 hours these males were able to inseminate only 0.05 females per male, and none of these females received a full complement of seminal fluid. Obviously, these males had lost the capacity for further insemination. The females used for these latter exposures were refractory for one of three reasons: immaturity, previous insemination or matrone injection. Males initially exposed to young virgin females, previously inseminated females or matrone-injected females were subsequently capable of inseminating 5.72, 5.70 and 5.61 females per male respectively. There was no indication of semen loss as a result of previous exposure to refractory females.

TABLE II

The effect of prior exposure to refractory females on male capacity for insemination. Each male was exposed to a different group of 10 females for each of 5 sequential 24 hour periods

No. ♂♂ tested	Types of refractory ♀♀ for exposure periods 1–3*	No. ♀♀ inseminated (of 10 available) per male in each period**				
		Refractory ♀♀			Mature virgin ♀♀	
		1	2	3	4***	5
33	None	– – – – – – no exposure – – – – – –			5.70 ± 1.24	0.05
29	Immature virgin	0	0	0	5.72 ± 1.36	0.17
28	Matrone-injected	0	0	0	5.61 ± 1.03	0.18
23	Previously inseminated	—	—	—	5.70 ± 1.29	0.13

* Follow exposure to refractory ♀♀ for periods 1–3, each ♂ was exposed to mature virgin ♀♀ for periods 4–5.

** Exposure periods, numbered 1–5, are 24 hours in length; exposure in gallon cage.

*** Mean ± S.D.

Coupling in gallon cages over a 24 hour period could not be readily observed, so the experiments reported in Table II were repeated using a lantern chimney as the mating chamber (Table III). Under these conditions, individual copulations were observed and timed. A single virgin male was exposed to five refractory females (24 hours old or matrone-injected) individually and in sequence. Two couplings of at least 8 seconds were permitted with each female, and the females were immediately examined for insemination or the presence of sperm on the external genitalia. The same male was then exposed in sequence to 10 receptive virgin females and permitted one copulation with each female. Immediately after coupling these females were also examined for insemination. The control experiments consisted of the exposure of a single virgin male to 10 virgin females in sequence, with one coupling per female. Thus, the entire process of testing one male required less than an hour.

As indicated in Table III, virgin males with no previous experience were able to inseminate an average of 6.5 females per male. Males after prior coupling with refractory females showed a similar, undiminished capacity. Males coupling first with young virgin or matrone-injected females inseminated an average of 6.5 and 6.4 females per male respectively. Again, coupling with refractory females had no effect on male insemination capacity. Moreover, there was no evidence of semen wastage after coupling. Of the 100 refractory females examined, not a single one had semen on the external genitalia.

The capacity for insemination of males exposed to females in gallon cages was slightly lower than the capacity of males exposed in lantern chimneys (about 5.7 *versus* 6.5). However, the mean capacities within experiments and between experiments were not significantly different. The results presented in Tables II and III are essentially identical.

TABLE III

The effect of prior coupling with refractory females on male capacity for insemination. Each male was permitted two couplings with each of five refractory females, then allowed one coupling with each of 10 virgin females.

No. ♂♂ tested	Type of refractory female	No. ♀♀ inseminated per male*	
		Refractory, 5 ♀♀ provided in sequence	Mature virgin, 10 ♀♀ provided in sequence**
10	—	----- no exposure -----	6.5 ± 0.97
10	Immature virgin	0	6.5 ± 1.27
10	Matrone-injected	0	6.4 ± 1.35

* All couplings observed in lantern chimneys. Each male exposed in sequence to 5 refractory and 10 mature virgin ♀♀.

** Mean ± S.D.

To support the conclusion drawn from Tables II and III, an entirely different type of experiment was designed. Radioactive males were produced by rearing larvae in a solution containing C^{14}. Groups of 15 ROCK females were exposed to 20 labelled males for a period of three days. Following exposure, females were anesthetized, crushed individually on a piece of filter paper, and placed in counting vials for radioactivity determination in the scintillation counter.

Level of radioactivity was determined for different kinds of females. Ten virgin females gave counts averaging 24.2 cpm; the average background was 25 cpm. Virgin females placed with labelled males gave much higher counts. In one experiment, 15 females gave individual counts ranging from 52.7 to 100.8, with a mean of 62.4 cpm. Females were examined for insemination prior to counting. In this experiment, all 15 females were inseminated. In another experiment, the following counts were obtained from 14 females: 20, 21, 46, 50, 57, 62, 65, 78, 92, 99, 102, 110, 114, 116. Dissection showed that the females with counts of 20 and 21 had not been inseminated. Thus, counts/minute of a given female readily indicated whether she had received semen from a male.

Table IV gives results of exposure of virgin and refractory females to labelled males. Males from two different strains, Rock and Distorter, were used. The Distorter strain gives sex ratio distortion, with high frequency of males in the progeny (Hickey and Craig, 1966). Virgin females exposed to males of either strain showed a high count, indicating insemination. On the other hand, refractory females gave counts very close to the average background count. In fact, the refractory females resembled the control, virgin females with no exposure to males. These females were refractory because they had previously been inseminated, and hence rendered monogamous by matrone. Clearly, the labelled males did not pass any seminal material to the refractory females.

Table IV

Radioactivity transferred to unlabelled ROCK females exposed for a 3-day period to virgin labelled males from two different strains

Isotope labelling of ♂♂*		Mean counts/minute/Rock ♀				
Material	Conc. in μc/ml	♀ virgin, no ♂ exposure (N = 10)	♀ exposed to ♂♂ (N = 15)			
			♀ virgin, mature		♀ refractory**	
			♂ Rock	♂ Distorter	♂ Rock	♂ Distorter
Isoleucine-C^{14}	0.05	24	62	83	28	26
Yeast Protein-C^{14}	0.10	25	53	52	28	28

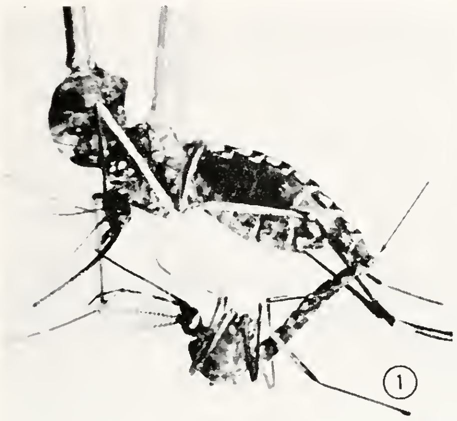
* See Table I.
** ♀♀ previously exposed to ♂♂.

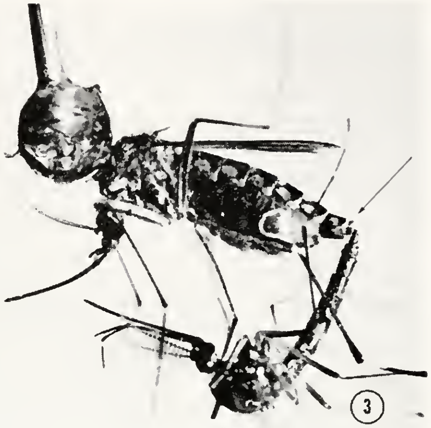
The results of the experiment with radioactive males (Table IV) fully confirm the earlier experiments (Tables II and III) which showed that prior exposure to refractory females did not diminish a male's capacity for insemination.

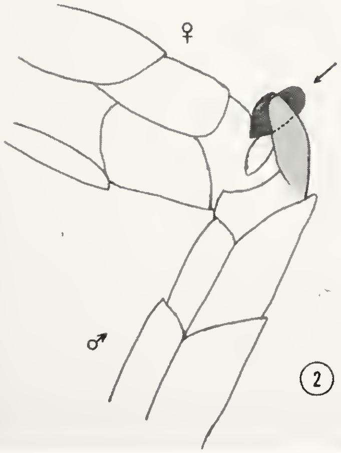
Observations of refractory and receptive behavior

Mating behavior of single females in suspended flight was observed with the aid of a stereoscopic microscope. Couplings resulting in insemination were markedly different from couplings by refractory females that did not result in insemination. Virgin females, 4 to 6 days old, were exposed to suspended flight to free-flying males; of 41 females tested, all but one were inseminated after a single coupling (Table V). Observations of these matings indicated that each coupling involved firm genital union and corresponded to the descriptions of copulation by Spielman (1964) and Jones and Wheeler (1965). Figure 1 pictures a male and female of *A. aegypti* engaged in copulation which resulted in insemination. The degree of genital contact of this successful copulation is illustrated in Figure 2.

The mean duration of genital contact for the 40 successful inseminations was 12.04 seconds (Table V). This average copulation time for receptive virgin females in suspended flight closely corresponds to the 12.1 second copulation time for receptive females in free flight in a lantern chimney. It would appear that suspension of the female had no discernable effect either on the duration of coupling or on the probability of insemination. Moreover, insemination of a receptive virgin female was normally achieved by the first male able to grasp her legs and bring his claspers into position for copulation. Indeed, if the terminalia of the male succeeded in touching the terminalia of the female, complete copulation and insemination was assured.







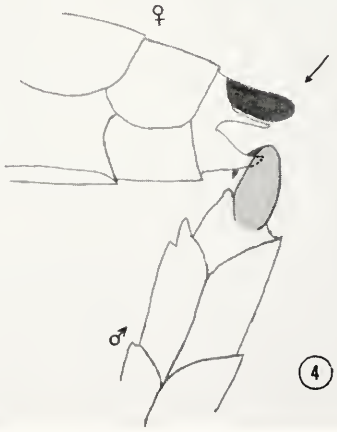


FIGURE 1. Copulation by a sexually mature female of *Aedes aegypti.* The arrow indicates the cerci of the female extending from beneath the claspers of the male. This copulation resulted in insemination of the female. The length of the male abdomen is approximately 3 mm.

FIGURE 2. Illustration of the degree of genital contact characteristic of copulation by a sexually receptive virgin female. The male claspers (shaded) fully engage the female cerci (dark).

FIGURE 3. Coupling without copulation by a sexually refractory female of *Aedes aegypti.* The arrow indicates the cerci of the female free and well above the claspers of the male. This coupling did not result in sperm transfer or insemination.

FIGURE 4. Illustration of the superficial genital contact typical of coupling by young refractory virgin females or previously inseminated females. The male claspers (shaded) do not touch the female cerci (dark). Copulation and insemination are impossible in this position.

Refractory females were exposed in suspended flight in the same manner as was employed with receptive females (Table V). Each refractory female was permitted a minimum of five couplings. None of 35 young tested actually copulated or were inseminated. Moreover, none of 25 matrone-injected females were inseminated. Obvious differences were noted in the behavior of refractory and receptive females. The duration of genital contact for couplings involving refractory females was extremely variable, with a range from 1 second to over 7 minutes; moreover, these copulations were never complete. Coupling by refractory females did not result in firm genital union (Figs. 3 and 4). The aedeagus of the male was so positioned that sperm transfer and insemination would be difficult if not impossible.

Visual determination of the state of receptivity of individual females is not difficult. An observer is able to differentiate between receptive and refractory females by noting the coupling activities of males. Males respond to sexually receptive females by ready contact, firm genital union and copulation. Males coupling with refractory females may be able to grasp the terminal sternite of the female but fail to copulate; they never achieve insertion of the aedeagus. These males may persist in their attempts to attain the proper coital position, but are never able to clasp the female cerci. The differences between the two types of coupling are so marked that females of unknown state can be determined as receptive or refractory on the basis of a single attempt if viewed with a stereoscope. However, to the unaided eye, both types of couplings appear to be normal copulations.

TABLE V

Receptivity to insemination of various types of females of Aedes aegypti *in suspended flight. Individual females were suspended by the mesonotum and induced to fly in the presence of males; each coupling was observed and timed.*

Female type	No. couplings allowed per ♀	No. ♀♀		Duration of genital contact in seconds
		Tested	Inseminated	
Mature virgin (4–6 days old)	1	41	40*	12.04 ± 3.30**
Matrone-injected	4–5	25	0	<6***
Saline-injected	1	13	13	10.41 ± 2.51**
Young virgin (22–24 hrs.)	4–5	35	0	<6***

* One unsuccessful coupling aborted at 6 seconds.

** Mean ± S.D.

*** Most couplings lasted less than 6 seconds; however, a few lasted over 60 seconds. Genital union was incomplete in all cases and no sperm was transferred.

Speed of onset of refractory behavior

In order to determine the speed with which females change from receptive to refractory coupling responses, individual females were observed under conditions of suspended flight. Each female was permitted two couplings with free-flying males. Each coupling was observed and timed, and the interval between the first and the second coupling was noted. Eighteen mature virgin females were tested.

The first coupling of each female was characterized by a firmness of genital union typical of a successful copulation. None of the second couplings were successful. The behavior of females during the second coupling was comparable to the behavior of previously inseminated or matrone-injected females. Moreover, these females were refractory to repeated attempts by several males.

The interval between the first and second couplings varied from 7 to 261 seconds with a mean of 93 seconds. This interval was not a function of female receptivity but was related to the speed with which the second male was attracted to the suspended female.

On the basis of these observations it would appear that the switch from receptive to refractory behavior is an almost instantaneous result of insemination. The first coupling invariably resulted in a firm copulation. The second coupling was always superficial and typically refractory. Moreover, the switch is not the result of the act of copulation itself. Virgin females will copulate repeatedly with semen-depleted males. Obvously, no semen transfer can occur, yet these copulations always show firm genital union, however, these females remain sexually receptive and will immediately copulate with a fresh male.

Discussion

Females of *A. aegypti* may couple repeatedly but they copulate only once, *i.e.*, semen is transferred only once. Consequently, they are monogamous. A female utilizes gametes from the insemination of a single male throughout her reproductive life. Only mature virgin females will copulate and can be inseminated. Both young virgin females and previously inseminated females are refractory to insemination; they may couple but do not copulate. Behavioral responses of the refractory female prevent firm genital union.

Spielman *et al.* (1967, 1969) felt that insemination of refractory females could not be prevented exclusively by behavioral factors. They felt that the condition of the bursa copulatrix of the female determined whether or not a female could be effectively inseminated. They froze females during and after forced-copulation and concluded that semen was transferred to females regardless of age or previous mating status. Only mature virgin females would retain the semen; refractory females ejected it from the bursa. Forcemated refractory females usually had internal masses of semen in the region of the genital atrium.

Unquestionably, refractory females can be inseminated by forced copulation (Craig, 1967; Akey and Jones, 1968; Gwadz and Craig, 1968). However the forced-copulation technique overrides the behavioral responses which prevent insemination under conditions of free flight. Therefore, data obtained using the forced-copulation technique are not relevant to normal insemination. We believe that our disagreement with the conclusions drawn by Spielman *et al.* (1967, 1969) are due in large part to their reliance on the forced-copulation technique.

Three lines of evidence from the present work bear out the contention that female behavior is adequate to prevent insemination of refractory females and that seminal expulsion is not involved. First, males exposed to refractory females showed no loss in insemination capacity. Males could inseminate 5–7 virgin females; repeated prior coupling with refractory females did not change this figure.

Secondly, refractory females exposed to males do not show external sperm masses. Of 100 refractory females examined, not a single one had semen on the external genitalia. Thirdly, refractory females placed with radioactive males showed no increase in radioactivity. If semen had been expelled from the bursa onto the external surface, the total count per female should have been raised.

From the standpoint of evolutionary parsimony, prevention of insemination of refractory females by seminal expulsion seems improbable. Considerable wastage of genetic material could occur. In our laboratory, Hausermann and Nijhout (in press) have shown that fecundity of male *A. aegypti* is strictly limited; a male cannot inseminate more than 5–7 females in a life-time. Yet, males of *A. aegypti* show hyperactive sexual activity (Roth, 1948), which would quickly lead to sperm depletion in the presence of refractory females. On the other hand, prevention of insemination by a behavioral mechanism of refractory females seems far more likely. If the female fails to give the proper cue indicating sexual receptivity, the male will not ejaculate and sperm will be conserved.

Spielman and his co-workers also argued that vacuolization of the bursal wall and spherule formation in the semen might be involved in the mechanism for semen retention. When seminal fluid comes in contact with a bursa *in vivo* or *in vitro*, physical changes do take place. However, there is no evidence to indicate that these events are in any way related to the retention of semen. Indeed, vacuolization and/or spherule formation are not always observed in females retaining semen (Spielman *et al.*, 1967, 1969).

Spielman concluded that the bursa of the mature virgin female (but not the immature or non-virgin female) has the capacity to "transform" seminal fluid and retain semen after copulation. However, Gwadz (1970) has shown that the copulatory behavior of female *A. aegypti* is regulated by the caudal sympathetic nervous system and is not related to the state of the bursa. When the terminal abdominal ganglion is surgically removed from a normally refractory female, she can be inseminated and will retain semen after copulation in free-flight. Although the chemical nature of the bursa of the young or previously inseminated refractory female is unchanged by the operation, the behavioral responses are altered. Normally refractory females that lack the terminal ganglion are not able to prevent copulation and insemination.

Sexual behavior of females of *A. aegypti* is similar in many respects to that displayed by females of the house fly, *Musca domestica*. Adams and Hintz (1969) have demonstrated a post-eclosion refractory period controlled by the corpora allata. Allatectomized female flies are mounted by males, but no insemination takes place because of female behavior. Moreover, house flies are monogamous; a component of the male seminal fluid renders females refractory to a second insemination for life (Riemann, Moen and Thorson, 1967). Sexually receptive females respond to courtship and mounting by extrusion of the ovipositor. This extrusion facilitates clasping and insemination by the male. Refractory females are mounted, but do not extrude the ovipositor; males are unable to clasp properly and cannot inseminate the female.

There are still serious gaps in our knowledge of mating behavior of female mosquitoes. The mode of action and precise target organs for the various hormones involved in regulating sexual behavior still need to be explored. Moreover, almost nothing is known about female pheromones, sex attractants and copulation initiators.

An even greater void exists in knowledge of the physiology of mating behavior of male mosquitoes. Because they are not disease vectors, males have reecived scant attention; their sex-related physiology has been virtually ignored.

Perhaps the greatest deficiency is in knowledge of mating behavior in the field. The where and when of mating, and the factors which bring the sexes together are questions of prime importance. The answers to these questions are especially relevant to those attempting programs of genetic control. These questions may prove to be among the most difficult to answer.

We wish to thank Dr. Andrew Spielman of the Department of Tropical Public Health, Harvard School of Public Health for his critical reading of this manuscript.

SUMMARY

1. Certain females of *A. aegypti* are refractory to insemination, either because they are too young or because of previous insemination. Earlier reports indicated that refractory females prevent insemination by expulsion of semen from the bursa copulatrix. The present work indicates that this is not the case.

2. Three lines of evidence indicate that refractory females prevent insemination by failing to give the cues that lead to male ejaculation. First, males exposed to refractory females show no loss in their ability to inseminate. Secondly, refractory females exposed to males show no evidence of external sperm masses resulting from expulsion. Thirdly, refractory females placed with radioactive males show no increase in radioactivity, indicating no transfer of labelled semen. Since the forced-copulation technique overrides normal behavior of females, earlier data obtained using this technique are not relevant to normal insemination.

3. Copulation by a mature virgin female is characterized by firm genital union followed by insemination. A refractory female allows the male to couple but not to copulate. Coupling involves a superficial joining of the genitalia; no ejaculation or insemination occurs.

LITERATURE CITED

ADAMS, T. S., AND A. M. HINTZ, 1969. Relationship of age, ovarian development, and the corpus allatum to mating in the housefly, *Musca domestica*. *J. Insect Physiol.*, **15**: 201–215.

AKEY, D. H., AND J .C. JONES, 1968. Sexual responses of adult male *Aedes aegypti* using the forced-copulation method. *Biol. Bull.*, **135**: 445–453.

CRAIG, G. B., JR., AND R. C. VANDEHEY, 1962. Genetic variability in *Aedes aegypti* (Diptera: Culicidae). I. Mutations affecting color pattern. *Ann. Entomol. Soc. Amer.*, **55**: 47–58.

FUCHS, M. S., G. B. CRAIG, JR. AND D. D. DESPOMMIER, 1969. The protein nature of the substance inducing female monogamy in *Aedes aegypti* *J. Insect. Physiol.*, **15**: 701–709.

FUCHS, M. S., G. B. CRAIG, JR. AND E. A. HISS, 1968. The biochemical basis of female monogamy in mosquitoes. I. Extraction of the active principle from *Aedes aegypti*. *Life Sciences*, **7**: 835–839.

GILLETT, J. D., 1955. The inherited basis of variation in the hatching-response of *Aedes* eggs (Diptera: Culicidae). *Bull. Entomol. Res.*, **46**: 255–265.

GWADZ, R. W., 1970. The neuro-hormonal regulation of sexual receptivity in female *Aedes aegypti* (Diptera: Culicidae). *Ph.D. thesis, University of Notre Dame,* 88 pp.

GWADZ, R. W., AND G. B. CRAIG, JR., 1968. Sexual receptivity in female *Aedes aegypti*. *Mosq. News,* **28**: 586–592.

GWADZ, R. W., AND G. B. CRAIG, JR., 1970. Female polygamy due to inadequate semen transfer in *Aedes aegypti*. *Mosq. News,* **30**: 355–360.

GWADZ, R. W., L. P. LOUNIBOS AND G. B. CRAIG, 1971. Precocious sexual receptivity induced by a juvenile hormone analogue in females of the yellow fever mosquito, *Aedes aegypti*. *Gen. Comp. Endocrinol.,* in press.

HAYES, R. O., 1953. Determination of a physiological saline solution for *Aedes aegypti* (L.). *J. Econ. Entomol.,* **46**: 624–627.

HICKEY, W. A., AND G. B. CRAIG, JR., 1966. Genetic distortion of sex ratio in a mosquito, *Aedes aegypti*. *Genetics,* **53**: 1177–1196.

JONES, J. C., AND R. E. WHEELER, 1965. An analytical study of coitus in *Aedes aegypti* (Linnaeus). *J. Morphol.,* **117**: 401–424.

HAUSERMANN, W., AND H. F. NIJHOUT, 1970. Loss of male fecundity following sperm depletion in *Aedes aegypti*. *J. Med. Entomol.,* in press.

LEA, A. O., 1968. Mating of virgin *Aedes aegypti* without insemination. *J. Insect Physiol.,* **14**: 305–308.

MACGREGOR, M. E., 1915. Notes on the rearing of *Stegomyia fasciata* in London. *J. Trop. Med. Hyg.,* **18**: 193–196.

RIEMANN, J. G., D. J. MOEN AND B. J. THORSON, 1967. Female monogamy and its control in houseflies. *J. Insect Physiol.,* **13**: 407–418.

ROTH, L. M., 1948. A study of mosquito behavior. An experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). *Amer. Midland Natur.,* **40**: 265–352.

SPIELMAN, A., 1964. The mechanics of copulation in *Aedes aegypti*. *Biol. Bull.,* **127**: 324–344.

SPIELMAN, A., SR. M. G. LEAHY AND V. SKAFF, 1967. Seminal loss in repeatedly mated *Aedes aegypti*. *Biol. Bull.,* **132**: 404–412.

SPIELMAN, A., SR. M. G. LEAHY AND V. SKAFF, 1969. Failure of effective insemination of young female *Aedes aegypti* mosquitoes. *J. Insect Physiol.,* **15**: 1471–1479.

Reference: *Biol. Bull.*, **140**: 215–229. (April, 1971)

ANALYSIS OF THE FEEDING RESPONSE IN THE ONUPHID POLYCHAETE *DIOPATRA CUPREA* (BOSC)[1]

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Like other annelids, spontaneous behavior of the onuphid polychaete *Diopatra cuprea* (Bosc) is dominated by rhythmic bursts of muscular activity that move large volumes of water through the tube. Unlike other species, however, the fraction of oxygen withdrawn in respiration is quite small. Considered in conjunction with our observation of a feeding response to chemical stimulation (Mangum and Cox, 1966), the low rate of oxygen withdrawal suggests that tube irrigation in this species is primarily a means of testing the medium for the presence of food, and only secondarily serves to ventilate the organs of respiratory exchange. When chemical stimuli are present in supra-threshold concentrations, the animal gives a behavioral response which concentrates food particles and culminates in ingestion (Mangum, Santos and Rhodes, 1968).

We have analyzed the response in terms of its separate behavioral components, and attempted to identify the substances in food which elicit the response.

MATERIALS AND METHODS

Behavioral observations

On several occasions, the response to fortuitous food stimuli has been observed in nature. However, analysis of the response was accomplished in the laboratory in running seawater, with controlled stimulation. Worms were initially observed in their natural opaque tubes mounted in the normal vertical position. Later observations were made on worms placed horizontally in glass tubes.

If observations are solely visual, some of the movements involved in the feeding response may be easily confused with other behavior patterns which generate water currents, *viz.* rejection of noxious chemical stimuli and ventilation movements. The similarity was also noted in *Chaetopterus variopedatus* by Wells and Dales (1951). Therefore, the nature of the response of *D. cuprea* to test substances was determined by visual observations in conjunction with quantitative measurements of the current (Fig. 1). The apparatus is similar to that used in investigations of ventilation rhythms (Dales, Mangum and Tichy, 1970; Mangum, 1971). The worm tube is attached to the recording chamber by the tail end, so that an upward deflection of the writing pen reflects current flow out of the tube. The direction and pattern of the current were determined by adding carmine particles to a test solution.

Each test substance was presented to a minimum of ten worms. Providing that the precautions below are taken, inconsistency of the response is not a problem. Although there is some variation in the intensity of the response given to supra-threshold stimuli, it is not sufficient to be confusing and it is not clearly correlated with intensity of stimulation. Therefore, we have treated the response as if it were all-or-none, which is very nearly correct. With the exception of the temperature dependence experiments, recalcitrance was rarely encountered within the period described below and designation of an acceptable criterion was unnecessary. In no case did the worms show less than 90% uniformity to qualitative tests, and 80% to quantitative tests.

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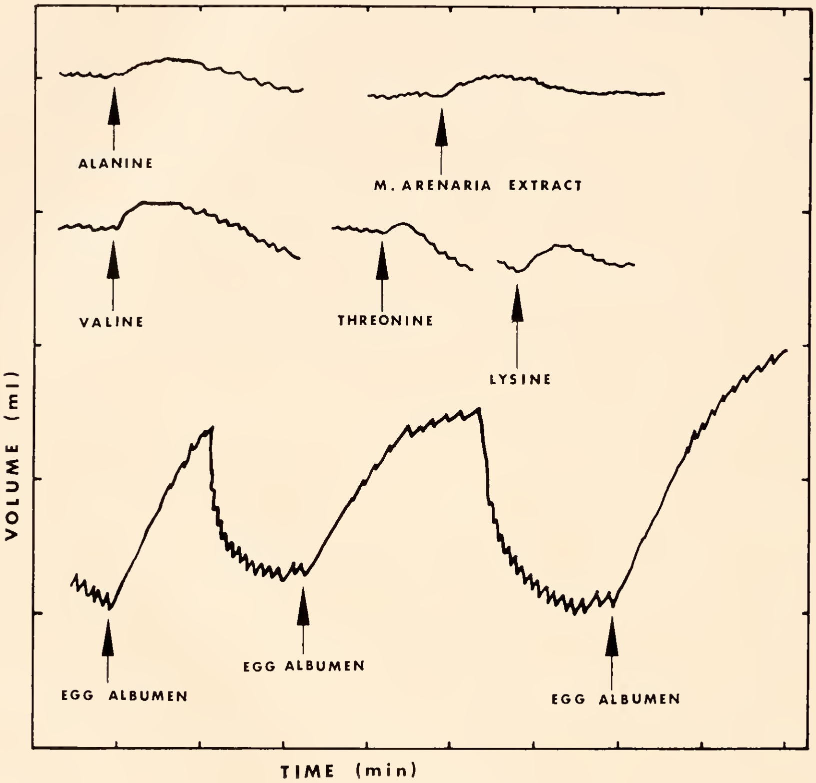

FIGURE 1. Currents generated by *Diopatra cuprea*. Feeding current given in response to clam extract and 10^{-2} *M* amino acid solutions. Rejection response given to egg albumen. Small deflections reflect ventilation movements. Each increment of ordinate is 1 ml; each increment of abscissa is 1 min.

All qualitative tests were made in running seawater, and ample time (ranging from 15 minutes to one hour, depending on the volume of the container) between tests was allowed to insure thorough renewal of the medium as well as subsidence of the response. It soon became apparent that the nutritional condition of the animal has a great influence on the response. In their natural habitat, worms have full guts during most months of the year (Mangum *et al.*, 1968). Animals brought into the laboratory may not respond, or they may respond inconsistently for a short period. Our observations have been made only on worms which have been acclimated to laboratory conditions for a minimum of three days. Conversely, very hungry worms respond to almost any stimulus; animals kept in the laboratory without feeding for six weeks or longer, depending on the temperature, have been observed attempting to feed from the surface film of the water overhead. In this case, the response is given continually. Therefore, tests were made only on animals collected less than two weeks previously in warm weather, and three weeks in colder weather. The worms were maintained without feeding in the laboratory, either in running seawater at 16–23° C or in filtered, recirculating seawater at 16–20° C.

Response to extracts of organisms

Extracts of a large number of marine animals were made by simply cutting open the animal and collecting the liquid which flowed into a beaker. The extracts were centrifuged, filtered and immediately tested. In a few cases, *viz.* the sponges and ectoproct, external pressure was applied in order to express fluid. Extracts of several species of algae and one higher plant were prepared by homogenization.

Subsequent to these initial tests, the response to clam extract was used as the criterion for a positive feeding response to chemical stimuli. Analyses of gut contents confirm the participation of at least one species of lamellibranch mollusc in the diet of *D. cuprea* (Mangum *et al.*, 1968). Valves of the soft-shelled clam *Mya arenaria* were bent back 180°, and the fluid (largely mantle fluid, muscle fluid and blood) collected. Some of the analyses were repeated on fluid from the clams *Mercenaria mercenaria, Tagelus plebius* and *Spisula solidissima,* as indicated below.

Analysis of clam extract

Centrifuged clam extract was dialyzed for 15 hours at 4° C against agitated seawater at a volume ratio of 1:1000, with a change of seawater every five hours. Dialysis was repeated on extracts of different individuals against running seawater at 21° C for 5 hours.

Clam extract (32 ml) was precipitated with 28 ml cold 10% (w/v) trichloroacetic acid, centrifuged and the supernatant fluid (pH initially 1.1) dialyzed against agitated standing seawater at 4° C until the pH reached that of seawater (12–16 hr).

Clam extract (25 ml) was precipitated first with an equal volume and then with $1\frac{1}{2}$ volumes of ethyl alcohol. The precipitate was washed three times with ethyl alcohol and then redissolved in a large volume of seawater. The solution was concentrated to the original volume of 25 ml by adding dry Sephadex G-25, and then tested.

A precipitate was also obtained by adding an equal volume of acetone, and then redissolved and tested. Additional acetone produced no precipitate.

Clam extract was chromatographed at 4° C on columns of Cal Bio-Gel P10 (1.5 × 20 cm) and Sephadex G-75 (1.5 × 30 cm). The gels were swollen and eluted in filtered seawater, and the fractions were collected with a LKB Ultrorac collector and drop counter (15 drops/ml). Positions on the P10 column were marked with a mixture of 1% (w/v) glycine and 0.1% Sephadex Blue dextran 2000. The glycine was detected with the ninhydrin reagent. The fractions were brought to room temperature and bioassayed.

The G-75 column (42 ml bed volume) was calibrated with 1.5% (w/v) recrystallized ovalbumin (Fisher Co.) and 3×10^{-4} M cytochrome *c* (Sigma Chemical Co.). Cytochrome *c* was detected visually; ovalbumin was detected by the Folin-Ciocalteau test (Clark, 1964). Blue dextran 2000 was used to determine the void volume. The pressure head was adjusted to yield a flow rate of 0.2 ml/min, which remained constant after initial equilibration of the column for 48 hr. Molecular weights of the standards were assumed to be 13,000 for cytochrome *c* and 45,000 for ovalbumin.

After initial separations on the G-75 column suggested that dilution of the sample on the column resulted in our failure to detect active substances by bioassay, clam extract was concentrated prior to chromatography. Dry Sephadex G-25 (water regain 2.5 ml/g) was placed in the bottom of a 10 ml glass syringe filled with a small piece of nylon net at the neck. Eight ml of clam extract were then pipetted into the gel, gently stirred and allowed to stand at 5° C for 45 min. Unabsorbed fluid (1.5 ml) was then forced out of the syringe up to the point of compression of the gel, and chromatographed as above on Sephadex G-75. Alternate fractions were subjected to the Folin-Ciocalteau test and the Molisch test (Clark, 1964), or bioassayed.

The amino acid content of extracts from *M. arenaria* and *M. mercenaria* was determined according to the procedure described by DuPaul and Webb (1970), using a Technicon Auto-Analyzer. The analyses were performed by Dr. K. L. Webb and Mr. W. D. DuPaul on extracts from three individuals of *M. mercenaria* and six of *M. arenaria,* acclimated to 18–20‰ salinity. Data from homogenates of tissue of *S. solidissima* have kindly been furnished by Mr. W. D. DuPaul.

Response to commercially prepared chemicals

A wide range of chemicals was tested at concentrations of 1% (w/v) in filtered seawater, unless otherwise specified. All of these substances were commercially prepared reagent grade chemicals, except for the protein globin, furnished by Dr. C. T. Grant.

Attention was then focused on the amino acids (Sigma Chemical Co.), initially assayed at concentrations of 10^{-2} M. Fresh solutions were made daily. The assay procedure was altered to determine the threshold concentration of each of eight amino acids. The worm, in a tube fabricated in the laboratory from clean sand and hence undecorated, was placed in a 5-inch finger bowl with a known quantity of filtered seawater. While constantly mixing with a magnetic stirrer, measured volumes of a 1 M solution of the amino acid were added quickly, to bring the total concentration of the medium to 10^{-7} M, and the response was noted. By adding further aliquots of the stock solution, the final concentration was raised in increments of a factor 10 until the response was positive. The experiment was repeated on a minimum of five worms. Unfortunately, the recording apparatus is not suitable for determinations of threshold concentrations, since it employs volumes of water in separate chambers which cannot be thoroughly mixed (Mangum, 1964). Therefore, the criterion for the feeding current component of the response was visual observation of anterior beating coupled with slow headward undulatory waves of muscular contraction. On several occasions we observed the initiation of beating unaccompanied by perceptible undulatory waves. We interpreted these movements as the spontaneous initiation of a rhythmic burst of irrigation activity, and, consequently, discarded the results.

A mixture of amino acids was prepared from the results of the analyses of clam extracts, and then bioassayed.

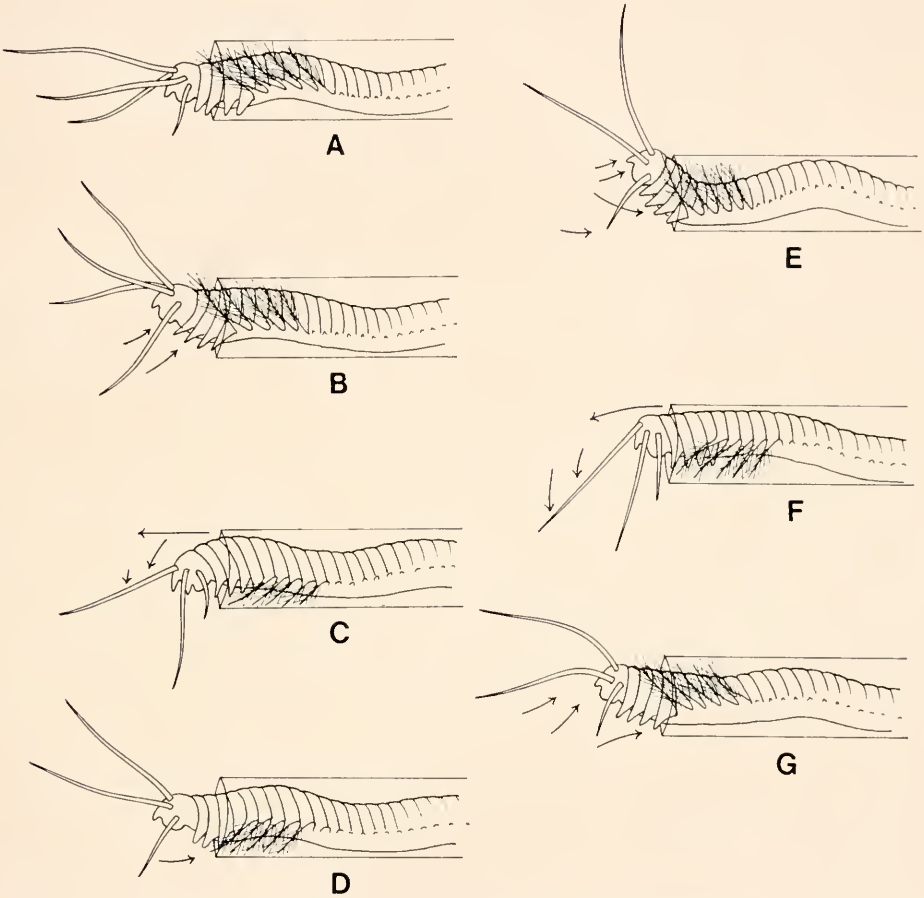

Figure 2. Movements involved in generating the feeding current.

Results

Description of the response

When a small piece of clam meat is placed within a few centimeters of the anterior opening of the tube, the worm extends its anterior region and undergoes searching behavior, usually strongly oriented toward the food. We have never observed failure to locate and ingest the food except at temperatures a few degrees higher than those at which the response is blocked (Mangum and Cox, 1966). At no time have we observed a feeding response to an organism held in the vicinity of the tube opening or to a member of the tube epibiota (Mangum *et al.*, 1968), if we were certain that the organism was intact. However, the response is given immediately to an organism which is only slightly punctured.

When a pipette of centrifuged, filtered clam extract is very gently emptied in the vicinity of the tube opening, the worm gives a charatceristic three-component response. It is this pattern of behavior which we have designated the feeding response and which has been the subject of our investigation. We have separated three components: the first rheotactic, the second chemotactic and the third both chemo- and mechano-tactic.

Initially, the antennae begin to rotate in their sockets, focused towards the source of the stimulus. Since antennal rotation can be elicited with many fluids, including seawater as well as clam extract, it cannot be considered a component of the chemically elicited response, but is instead a rheotactic component. The second component, which usually follows the first in time, consists of a set of movements which generate a current (Fig. 1) and an eddy at the tube aperture. The anterior end of the worm beats dorsoventrally against the sides of the tube, creating a centripetal eddy which concentrates particulate matter in the immediate vicinity. Simultaneously, an undulatory wave of contraction of the body wall muscles, also dorsoventral, passes very slowly in the anterior direction. Since the ventral beat of the anterior region is followed by a slight pause, there is a leak from the eddy in the dorsal direction, bringing concentrated particles into contact with the mouth region (Fig. 2). Because of the undulatory wave, the net flow of the feeding current is outwards from the tube (Fig. 1), and the tube is cleared of potential fouling material. We have been able to elicit this component of the response only with chemical stimuli. It is easily confused with normal tube irrigation, the movements differing only in the much slower rate of passage of the undulatory wave and, consequently, the velocity of the current (Fig. 1). The feeding current movements also resemble those of the rejection response, which is accompanied by an extremely rapid headward undulation and, thus, a far greater volume moved (Fig. 1), as well as withdrawal to a deeper position in the tube.

The third and final component, which may occur simultaneously with the second, consists of two sets of movements: (1) Parapodial clasping, in which the greatly enlarged parapodia of the anterior segments move ventrolaterally, with the result that the worm grasps large particles concentrated in the eddy. If an object is grasped, the movements cease; if not, they continue. (2) Jaw flexing and mouth opening, with the result that the worm tears off a piece of food held by the parapodia and ingests it. The two sets of movements are invariably coupled; one does not occur without the other. This component can be elicited by either chemical or mechanical stimuli. It is given in response to a jet of clam extract, a piece of clam meat or a small piece of congealed rubber cement gently touching the tentacles or palps. Unlike clam meat, the worm grasps rubber cement, ingests it and soon regurgitates it. After a few minutes, the worm will take another piece of rubber cement, regurgitate it, and take another for at least eight trials.

If alternate pieces of clam meat and rubber cement are fed, however, the worm no longer responds to rubber cement after five or six pieces of clam meat, whereas clam meat is still an effective stimulus. Although the state of satiety apparently

TABLE I

*Response to extracts of marine organisms**

Species	Response
Porifera	
Microciona prolifera	N.R.
Halichondria sp.	N.R.
Coelenterata	
Metridium senile	F.C.
Diadumene leucolena	N.R.
Sipunculida	
Golfingia gouldi	F.C.
Annelida	
Amphitrite ornata	F.C., M.O.
Hydroides dianthus	F.C.
Nereis succinea	F.C., M.O.
Diopatra cuprea	F.C., M.O.
Arenicola marina	F.C., M.O.
Glycera dibranchiata	F.C., M.O.
Mollusca	
Ensis directus	F.C.
Aequipecten irradians	F.C.
Mya arenaria	F.C., M.O.
Mercenaria mercenaria	F.C., M.O.
Crassostrea virginica	F.C., M.O.
Tagelus plebeius	F.C., M.O.
Nassarius obsoletus	F.C., M.O.
Polinices duplicatus	F.C.
Spisula solidissima	F.C., M.O.
Arthropoda	
Homarus americana	F.C.
Carcinus meanas	F.C.
Artemia salina	F.C., M.O.
Ectoprocta	
Bugula turrita	Rej.
Echinodermata	
Asterias forbesi	F.C.
Arbacia punctulata	F.C.
Strongylocentrotus drobachiensis	F.C.
Echinarachinius parma	F.C.
Urochordata	
Ciona intestinalis	Rej.
Vertebrata	
Fundulus fundulus	F.C.
Chlorophyta	
Codium sp.	F.C.
Rhodophyta	
Porphyridium sp.	N.R.
Angiosperm	
Zostera sp.	F.C.

* N.R. = no response; F.C. = feeding current; M.O. = mouth opening; Rej. = rejection.

exerts a differential influence on the response to clam meat and rubber cement, the pattern of the response in a hungry worm is indistinguishable.

The location of the receptors mediating chemically stimulated components of the response is uncertain. Although the worm is lavishly provided with anterior protrusions of various sorts, they seem to be unessential to the initiation of the response. After successive surgical removal of each of the five occipital antennae, including ceratophores, both sets of palps and several gills, the worm continues to give both components of the chemically stimulated response. Even after removal of the prostomium and several anterior setigers, the worm still generates a current of similar magnitude as the feeding current, if clam extract is applied. Indeed, a localized muscular contraction of the body wall is elicited by directing a jet of clam extract at the dorsal surface of the isolated autotomized tail region. Finally, Sven Donaldson and James Case (personal communication from Dr. James F. Case, University of California, Santa Barbara) have recorded electrical potentials from the segmental nerves of several isolated postbranchial segments, when clam extract was applied.

TABLE II

Molar concentration of free amino acids in three species of lamellibranch molluscs

Amino acid	*Mercenaria mercenaria* extract (N = 3)	*Mya arenaria* extract (N = 6)	*Spisula solidissima* (gill tissue) (N = 4)
Aspartic acid	7×10^{-8}	1×10^{-4}	5×10^{-5}
Taurine	7×10^{-4}	7×10^{-5}	2×10^{-4}
Glycine	2×10^{-4}	5×10^{-4}	2×10^{-5}
Histidine	2×10^{-5}	4×10^{-5}	1×10^{-5}
Threonine	4×10^{-5}	8×10^{-5}	3×10^{-6}
Alanine	1×10^{-4}	3×10^{-4}	5×10^{-5}
Isoleucine	6×10^{-6}	2×10^{-6}	1×10^{-6}
Leucine	1×10^{-5}	4×10^{-6}	1×10^{-6}
Lysine	5×10^{-5}	5×10^{-5}	4×10^{-6}
Arginine	4×10^{-5}	4×10^{-5}	7×10^{-6}
Glutamic acid	1×10^{-7}	7×10^{-5}	3×10^{-5}
Serine	5×10^{-6}	3×10^{-4}	7×10^{-6}
Tyrosine	1×10^{-5}	2×10^{-5}	1×10^{-5}
Cysteine	none	5×10^{-6}	4×10^{-6}
Tryptophan	none	$>1 \times 10^{-6}$	none
Ornithine	1×10^{-8}	7×10^{-5}	1×10^{-6}
Proline	2×10^{-5}	1×10^{-5}	none
Valine	1×10^{-5}	3×10^{-6}	1×10^{-5}
Methionine	6×10^{-6}	1×10^{-5}	3×10^{-7}
Phenylalanine	2×10^{-6}	1×10^{-5}	1×10^{-5}

Temperature sensitivity of the response

Animals collected at 3–8° C and acclimated in the laboratory to 5° for two weeks give the response to clam extract only rarely. Of 20 worms tested, 15 gave no response to either clam extract or to clam meat gently applied to the tentacles. Five worms produced a current somewhat in excess of normal spontaneous irrigation but none gave the mouth opening component. Only one in 20 ingested the clam meat. In contrast, 18 of 20 animals acclimated to 10° C for two weeks gave the complete response to clam extract.

Low temperature blockage of the response continues for many weeks; ultimately, however, inhibition is released. Of eight animals kept at 5° C in the laboratory without food for seven months, six ingested pieces of clam meat placed in the vicinity of the tube opening.

Response to extracts of organisms

The results of an assay of 32 marine organisms for the presence of substances eliciting the feeding response in *D. cuprea* are given in Table I. Many of the tests were made prior to analysis of the response into its separate components when the criterion was a recorded current of the correct magnitude together with visual observation of the movements. In all cases where mouth opening is not reported, we do not know whether it occurs. However, we have never been able to confirm the occurrence of the feeding current component without mouth opening in response to a freshly prepared natural extract.

TABLE III

Response of Diopatra cuprea *to various substances**

Substance	Response	Substance	Response
β-D-Glucose, 1%	F.C.	Ethyl alcohol	Rej.
α-D-Glucose, 1%	N.R.	Distilled water	Rej.
D-Galactose, 1%	F.C.	Vegetable oil emulsion	F.C.
α-Lactose, 1%	F.C.	Mineral oil emulsion	F.C.
β-Lactose, 1%	F.C.	Butyric acid, 1%	Rej.
D-Arabinose, 1%	N.R.	Linoleic acid, 1%	N.R.
Maltose, 1%	F.C., M.O.	Trichloroacetic acid, 1%	Rej.
L-Rhamnose, 1%	N.R.	Reduced glutathione, 10^{-5} M	N.R.
D-Mannose, 1%	N.R.		
D-Xylose, 1%	F.C.	Gelatin, 1%	N.R.
D-Sorbitol, 1%	F.C.	Casein, 1%	N.R.
Sucrose, 1%	N.R.	Trypsin, 0.1%	F.C.
Glycerol, 1%	N.R.	Trypsin, 1%	Rej.
Glycogen, 1%	N.R.	Trypsin, 1%, boiled	Rej.
Inulin, 1%	N.R.	Egg yolk	F.C.
N-Acetyl glucosamine, 1%	F.C., M.O.	Egg albumen	Rej.
N-Acetyl galactosamine, 1%	F.C., M.O.	Clam extract, boiled	Rej.
Hemoglobin (Fisher Co.), 1%	F.C., M.O.	Crab urine	Rej.
		Urea, 1%	F.C.
Human globin, 10^{-11} M	F.C.	Hydroxyurea, 1%	F.C.
Arenicola marina blood, fresh	F.C., M.O.		
A. marina blood, aged 24 hr at 5° C	Rej.		

* F.C. = feeding current; M.O. = mouth opening; Rej. = rejection; N.R. = no response.

The condition of the extract certainly determines the nature of the response. While either freshly prepared or frozen and thawed clam extract causes the feeding response, boiled and cooled clam extract elicits a rejection response. Whole blood freshly collected from the polychaete *Arenicola marina* causes the feeding response, but 24 hour-aged blood which shows methemoglobin absorption bands elicits a rejection response.

D. cuprea responds to an extract of almost any organism, including itself. The absence of a response to the two sponges and to the red alga *Porphyridium* may reflect the difficulty of obtaining fluid rather than the absence of active substances. Lack of a response to extract of the anemone *Diadumene leucolena* cannot be similarly explained, however.

Response to commercially prepared chemicals

The response to commercially prepared chemicals is not highly correlated with molecular structure. For example, *D. cuprea* gives a feeding current response to a number of sugars and fails to do so to others (Table III). The activity of test substances is not related to chemical properties in any way which is obvious to us. Neither the optical activity of hydroxyl groups, nor the state of polymerization, nor the reducing properties of the molecules explain the distribution of activity.

TABLE IV

Response of Diopatra cuprea *to amino acids**

Amino acid	Response	Amino acid	Response
Aspartic acid	Rej.	Alanine	F.C.
Glutamic acid	Rej. (10^{-5} *M*)	Threonine	F.C.
Taurine	N.R.	Lysine	F.C.
Histidine	N.R.	Cysteine	F.C. (10^{-6} *M*)
Glycine	N.R.	Asparagine	F.C.
Cystine	N.R.	Methionine	F.C. (10^{-5} *M*); M.O. (10^{-3} *M*)
Isoleucine	F.C.	Phenylalanine	F.C. (10^{-6} *M*); M.O. (10^{-2} *M*)
Leucine	F.C.	Proline	F.C.; M.O.
Tryptophan	F.C. (10^{-5} *M*)	Hydroxyproline	F.C. (10^{-7} *M*); M.O.
Tyrosine	F.C. (10^{-7} *M*)	Valine	F.C. (10^{-8} *M*); M.O. (10^{-4} *M*)
Serine	F.C. (10^{-5} *M*)	Norvaline	F.C.
Arginine	F.C.	Norleucine	F.C.
Ornithine	F.C.; M.O.		

* Rej. = rejection; N.R. = no response; F.C. = feeding current; M.O. = mouth opening; 10^{-n} *M* = threshold concentration.

Although the worms do give the feeding current response to oil emulsions, which cannot be due to mechanical stimulation, lipids as a class would seem to be unlikely candidates for active substances in natural extracts. Several proteins elicit a response, although not all of those tested.

The worms give at least the feeding current component to 20 of 25 amino acids tested, and the complete response to six (Table IV). Once again, there does not seem to be a common denominator in terms of chemical properties of the amino acids which do or do not give a response, or which elicit only the feeding current component. The threshold concentrations for the feeding current component seem rather low, and those for mouth opening moderately high. Most importantly, the threshold for the feeding current lies below the figure for the concentration of four amino acids in *M. mercenaria* extract (Table II), six in *M. arenaria* extract

(Table II), six in *S. solidissima* gill homogenates (Table II), and in *S. solidissima* muscle homogenates (personal communication from W. D. DuPaul, Virginia Institute of Marine Science). The threshold for mouth opening is attained by only one amino acid, valine, in *M. arenaria* muscle.

When a mixture of 20 amino acids was prepared according to the recipe for *M. arenaria* extract (see below) given in Table II, 10 out of 10 worms tested gave a feeding component response to the solution. But 8 out of 10 failed to give the mouth opening component. None of the worms gave a rejection response, despite the supra-threshold concentration of glutamic acid. Therefore, synergistic effects are not completely absent.

Analysis of clam extract

When *M. arenaria* extract is diluted to 10^{-2}, the worm still gives all three components of the response. When it is further diluted to 10^{-3}, however, mouth opening no longer occurs. Dialysis of extracts of four species of lamellibranch molluscs yielded an interesting dichotomy: the non-dialyzable fraction from *Mya arenaria, Spisula solidissima* and *Tagelus plebius* elicited a complete feeding response, whereas that from *Mercenaria mercenaria* gave no response. A sample of each extract aged at the same temperature for the same length of time gave a complete response. The results were identical, regardless of the temperature or conditions of dialysis.

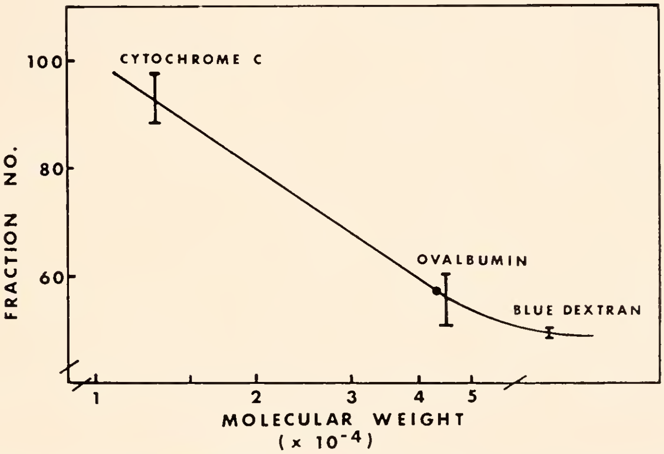

FIGURE 3. Calibration of Sephadex G-75 column. Closed circle (●) indicates position of unknown active substance.

Trichoroacetic acid precipitation of *M. arenaria* and *T. plebius* extracts followed by dialysis failed to alter the results. The non-dialyzable and non-precipitable fractions elicited the feeding response.

The redissolved ethyl alcohol precipitate from *M. arenaria* gave the feeding current component but not mouth opening; the redissolved acetone precipitate gave no response.

When the extract of *M. mercenaria* was chromatographed on Cal Bio-gel P10, only the fraction corresponding to the amino acid marker elicited a response, and only the feeding current component was given. Chromatography of *M. arenaria* extract yielded an active fraction corresponding to the amino acid marker, and also a second active fraction 1–3 ml behind the void volume marker. The worms gave a complete response to both fractions.

Because the larger molecule was eluted so close to the void volume on Bio-gel P10 subsequent analyses were made on a more precisely calibrated column of Sephadex G-75 (Fig. 3). When Sephadex G-25 concentrated extract of *M. arenaria* was chromatographed on the G-75 column, the worms gave a complete response to fraction numbers 123–131, corresponding to the glycine marker, and also to numbers 57–58, suggesting a molecular weight of 45,000 if the substance behaves like the globular protein markers. However, the fractions gave a strong positive reaction to the Molisch test for carbohydrate as well as a weaker positive reaction to the Folin-Ciocalteau test (Clark, 1964). These results, in the light of elution of the second active substance behind the blue dextran marker on Bio-gel P10, suggest that the unknown substance is a glycoprotein. Glycoproteins do not behave on gel columns like ideal globular proteins (Whitaker, 1963; Andrews, 1965; Ward and Arnott, 1965). The results of Ward and Arnott (1965) suggest that the departure depends upon the ratio of carbohydrate to protein, which is not known for the glycoprotein in clam extract. However, the molecular weight of clam extract glycoprotein may be less than 20,000, since it was retarded by Bio-gel P10, and possibly considerably less since it was not precipitated by trichloroacetic acid. The manufacturers give the figure of 10,000 as the exclusion limit for Bio-gel P10, and 5000–17,000 as the effective operating range. It is possible that the failure to be precipitated by TCA is due to a high ratio of carbohydrate to protein.

The concentration of amino acids in our extract of *M. mercenaria,* and *M. arenaria,* and in tissue homogenates of *S. solidissima* is given in Table II. In *M. mercenaria* extract, the four amino acids serine, tyrosine, valine and phenylalanine occur in concentrations exceeding the threshold for the feeding current component. None of the amino acids occurs in concentrations exceeding the known threshold values for mouth opening.

In our *M. arenaria* extract, six amino acids occur in concentrations above the threshold for the feeding current component, but none exceed that for mouth opening. The six are the same as those in *M. merceniara,* with the addition of cysteine and methionine.

S. solidissima gill homogenates contain concentrations of the four amino acids tyrosine, cysteine, valine and phenylalanine equal to or in excess of threshold for the feeding current component of the response.

Data from several other sources are pertinent. Carr's (1967b) determination of the amino acid content of extract from the shrimp *Peanaeus duorarum* predicts that it will elicite the feeding current component in *D. cuprea,* since valine and tyrosine occur in supra-threshold concentrations. Stephens and Preston (University of California at Irvine, personal communication) measured amino acid content of coelomic fluid of the polychaete *Glycera dibranchiata,* which is also known to elicit the response (Table I). Based on their data, the feeding current component may be explained by supra-threshold concentrations of six amino acids, and mouth opening to the presence of 2×10^{-3} M concentrations of valine. Finally, the results of DuPaul and Webb (1970) on *M. arenaria* (20‰ salinity) suggest that the feeding current response to muscle homogenates can be explained by suprathreshold concentrations of tyrosine, tryptophan, valine, phenylalanine, serine and cysteine. None of these occurs in threshold concentrations for mouth opening, and in fact the worm does not respond with this component to the preparation. Gill homogenates contain the first four amino acids in excess of threshold for the feeding current (personal communication from W. D. DuPaul and K. L. Webb, Virginia Institute of Marine Science). The concentration of valine ($2\text{–}7 \times 10^{-4}$ M) explains mouth opening as well.

Discussion and Conclusions

Chemically mediated feeding responses by annelids to natural extracts were studied by Copeland and Wieman (1924), who reported that *Nereis virens* gives a highly oriented response, culminating in ingestion, to extracts of crustaceans, lamellibranchs and gastropods. They also pointed out the importance of burrow irrigation in permitting the animal to test the medium for chemical stimuli. A response to crab extract by *Onuphis eremita* was noted by Tampi (1959). In most cases of chemically mediated feeding responses in aquatic invertebrates, it is not known whether the extract derives from an organism which comprises part of the natural diet. Nonetheless, these investigations are at least suggestive of the behavioral mechanism which allows the animal to recognize its food.

Identification of active substances has been attempted in the past two decades by analyzing responses of various animals to commercially prepared chemicals. Most investigators have assumed that good candidates for the role of active substances are of small size, readily diffusible, low in density, and active in fairly low concentrations which may be expected to occur in nature. Amino acids and small peptides meet the physical requirements, and they are also believed to be widespread in animal fluids, although little quantitative information has been available until recently. Consequently, feeding responses of various animals to test solutions of amino acids and small peptides have been reported (Loomis, 1955; Fulton, 1963; Carr, 1967a, 1967b; Crisp, 1967; Lindstedt, Muscatine and Lenhoff, 1968; Blanquet and Lenhoff, 1968). To evaluate the significance of these very interesting behavioral responses, one crucial question must be answered: does the threshold concentration of the particular substance actually occur in extracts of food which elicit a response? Unfortunately, there have been few reports of amino acid or peptide concentrations in test extracts. Suitability of the test extract has also been inadequately considered: does it originate from a source which is likely to resemble the animal's food? In the present case, the response to 10^{-5} M methionine is of some interest to us, but there is no evidence to suggest that it is of any interest to *Diopatra cuprea.*

To our knowledge, the only investigation in which extensive quantitative analysis of natural extracts has been undertaken in conjunction with the response to commercially prepared chemicals is that of Carr (1967a, 1967b), dealing with a graded response of the mud snail *Nassarius obsoletus* to shrimp extract. As a result of an elaborate analytical procedure, Carr (1967b) succeeded in preparing a quantitative solution of 12 compounds which duplicates 50% (probit scale) of the response to shrimp extract.

The feeding response of *D. cuprea* seems to be far less exacting, a quality which is possibly of adaptive significance to a sedentary animal that obtains its food by a less direct mechanism. Absence of specificity to commercially prepared chemicals is certainly supported by evidence of a wide range of foods found in the gut (Mangum *et al.,* 1968). Unfortunately, the organisms eaten by natural populations of *D. cuprea* are too small to yield copious extracts for laboratory analysis. A truly satisfying account of a chemically mediated feeding response would involve the demonstration of supra-threshold concentrations of at least one active substance in each kind of food taken by the species in nature. We have certainly not achieved such a level of explanation. But we do know that one component of the response is given to substances which occur in supra-threshold concentrations in extracts of several lamellibranch molluscs. And that the lamellibranch *Gemma gemma* is found in the guts of worms taken in nature (Mangum *et al.,* 1968).

The animal's food in nature may often be particulate in form (Mangum *et al.,* 1968). If the feeding current component is elicited by valine emanating from food particles, for example, then mouth opening and ingestion will be elicited by mechanical stimulation from the particles concentrated by the feeding current. Thus the process of feeding can be attributed to the presence of four amino acids in *M. mercenaria* extract or 6 in *M. arenaria,* with considerable margin to spare. Nonetheless, the observed response to chemical stimulation from *M. arenaria* extract includes the mouth opening component. The larger active molecule, apparently a glycoprotein, may be essential to understanding this component of the observed response.

We are indebted to Mr. Robert A. Graham for his contributions to our understanding of the response.

LITERATURE CITED

Andrews, P., 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.,* **96**: 595–606.

Blanquet, R. S., and H. M. Lenhoff, 1968. Tyrosine enteroreceptor of *Hydra:* its function in eliciting a behavior modification. *Science,* **159**: 633–634.

Carr, W. E. S., 1967a. Chemoreception in the mud snail, *Nassarius obsoletus.* I. Properties of stimulatory substances extracted from shrimp. *Biol. Bull.,* **133**: 90–105.

Carr, W. E. S., 1967b. Chemoreception in the mud snail, *Nassarius obsoletus.* II. Identification of stimulatory substances. *Biol. Bull.,* **133**: 106–127.

Clark, J. M., 1964. *Experimental Biochemistry.* W. H. Freeman and Co., San Francisco, California, 228 pp.

Copeland, M., and H. L. Wieman, 1924. The chemical sense and feeding behavior of *Nereis virens* Sars. *Biol. Bull.,* **47**: 231–238.

Crisp, D. J., 1967. Chemoreception in cirripedes. *Biol. Bull.,* **133**: 128–140.

Dales, R. P., C. P. Mangum and J. C. Tichy, 1970. Effect of changes in oxygen and carbon dioxide concentrations on ventilation rhythms in onuphid polychaetes. *J. Mar. Biol. Ass. U. K.,* **50**: 365–380.

DuPaul, W. D., and K. L. Webb, 1970. The effect of temperature on salinity-induced changes in the free amino acid pool in *Mya arenaria. Comp. Biochem. Physiol.,* **32**: 785–801.

Fulton, C., 1963. Proline control of the feeding reaction in *Cordylophora. J. Gen. Physiol.,* **46**: 823–837.

LINDSTEDT, K. J., L. MUSCATINE AND H. M. LENHOFF, 1968. Valine activation of feeding in the sea anemone *Boloceroides*. *Comp. Biochem. Physiol.,* **26**: 567–572.

LOOMIS, W. F., 1955. Glutathione control of specific feeding reactions of hydra. *Ann. New York Acad. Sci.,* **62**: 209–228.

MANGUM, C. P., 1964. Activity patterns in metabolism and ecology of polychaetes. *Comp. Biochem. Physiol.,* **11**: 239–256.

MANGUM, C. P., 1970. Respiratory physiology in annelids. *Amer. Sci.,* **58**: 641–647.

MANGUM, C. P., AND C. D. COX, 1966. A feeding response to chemical stimuli in the onuphid polychaete *Diopatra cuprea*. *Amer. Zool.,* **6**: 456–457.

MANGUM, C. P., S. L. SANTOS AND W. R. RHODES, 1968. Distribution and feeding in the onuphid polychaete, *Diopatra cuprea* (Bosc). *Mar. Biol.,* **2**: 33–40.

TAMPI, P. R. S., 1959. An ingenious method of collecting polychaete worms for fish bait. *J. Mar. Biol. Ass. India,* **1**: 250–251.

WARD, D. N., AND M. S. ARNOTT, 1965. Gel filtration of proteins with particular reference to the glycoprotein, luteinizing hormone. *Anal. Biochem.,* **12**: 296–302.

WELLS, G. P., AND R. P. DALES, 1951. Spontaneous activity patterns in animal behaviour: the irrigation of the burrow in the polychaetes *Chaetopterus variopedatus* Renier and *Nereis diversicolor* O. F. Müller. *J. Mar. Biol. Ass. U. K.,* **29**: 661–680.

WHITAKER, J. R., 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. *Anal. Chem.,* **35**: 1950–1953.

Reference: *Biol. Bull.*, **140**: 230–241. (April, 1971)

EFFECTS OF PRENATAL X-IRRADIATION ON DEVELOPMENT AND POSTNATAL VIABILITY OF INBRED AND HYBRID MICE[1]

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Studies of the effects of prenatal x-irradiation of mammals have emphasized in recent years long-term pathological and functional investigations. These investigations have included studies on growth (Murphree and Pace, 1960; Nash and Gowen, 1962; Parish, Murphree and Hupp, 1962; Rugh, Duhamel, Chandler and Varma, 1964), reproduction (Russell, Badgett and Saylors, 1960; Rugh and Jackson, 1958; Nash, 1969), and postnatal mortality and lifespans (Nash and Gowen, 1965; Rugh and Wohlfromm, 1965; Sikov, Resta and Lofstrom, 1969).

In spite of the obvious significance to human populations, relatively little work has been done to explore the influence of genetic background in conditioning the response of embryos to x-irradiation. Most of the earlier studies either have utilized animals of unknown heterogeneous origin or single strains of uniform origin. A few studies have demonstrated the significance of genetic variability in embryonic response (Rugh, 1958; Callas, 1962; Nash and Gowen, 1962, 1965; Dagg, 1963; Nash, 1969).

The recent review of Brill, Nance, Engel, Glasser, and Forgotson (1967) has pointed out the basic similarities between laboratory animal studies and long-term, postnatal effects observed in humans that have been exposed to prenatal irradiation. The studies reported in the present paper were designed to examine effects of prenatal x-irradiation upon the responses of inbred and hybrid mouse embryos as measured by morphological changes and changes in postnatal viability.

MATERIALS AND METHODS

The mice that were used in these studies were obtained from first pregnancies of matings within and among three inbred strains: BALB/Gw, K/Gw and S/Gw. Descriptions of the characteristics of the strains are provided in previous papers (Nash and Gowen, 1962, 1965). The matings produced progeny which were either inbred or hybrid, and provided nine different inheritance types.

Mated females were examined daily for the presence or absence of a vaginal plug, the plug being the sole criterion used to time the period of gestation and the approximate age of embryos at irradiation. The afternoon of the day on which the plug was observed was considered to be day ½ of gestation. Females were irradiated at 4 PM, and the gestational ages of the embryos at the time of treatment were considered to be approximately 6½, 10½, 14½ or 17½ days. Developmentally, these ages correspond to a time shortly after implantation, a period of major organogenesis, a period of minor organogenesis, and a time of rapid fetal growth, respectively. However, there are a number of factors which are operative which make even the above approximate estimates of developmental stages subject to error. There is considerable variation not only between litters, but also among individuals of the same litter, as to fertilization time, implantation time, and subsequent developmental time. Allen and MacDowell (1940), for example, observed that embryos of the same chronological age may differ as much as 24 hours in developmental age.

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The source of irradiation was a General Electric Maxitron which was operated at 250 pkv, 30 ma with 0.25 mm Cu + 1 mm Al filtration at a distance of 50 cm from anode to mid-mouse. The dose rate was approximately 133r/minute. Pregnant mice were exposed to single doses of whole-body irradiation of 0, 20, 80, 160, or 320 roentgens.

TABLE I

Percentage of developmental abnormalities induced by irradiation at $10\frac{1}{2}$ days gestation in inbred and hybrid mice

	Morphological characters observed at birth					
	80r		160r		320r	
	Inbred	Hybrid	Inbred	Hybrid	Inbred	Hybrid
Number of animals observed	72	76	22	48	17	33
Abnormal	7	3	64	29	100	100
Eyelids open	1	0	9	2	6	12
Cranial blister	0	1	14	2	100	85
Vaulted cranium	0	0	0	2	24	6
Abnormal tail	6	1	41	10	100	100
Overgrowth of feet						
Forefeet	0	0	5	0	0	0
Hind feet	0	0	23	10	12	15
Reduction of feet						
Forefeet	0	0	5	4	65	70
Hind feet	0	0	0	2	59	67

Mice were examined twice daily so that newborn litters usually were found within 12 hours of birth. Newborns were given a gross, external morphological examination. Progeny were checked daily for deaths and all mice were maintained at least through 75 days of age. Litters remained with their treated dams through the suckling period. All mice were weaned at 30 days, and the males and females were separated at that time.

Additional data from these experiments describing effects on postnatal growth, reproductive performance, and lifespans are provided in several previous papers (Nash and Gowen, 1961, 1962, 1963a, 1963b, 1965; Nash, 1969).

RESULTS

Morphology

The only three treatments which yielded abnormalities in more than singular occurrence were doses of 80, 160, or 320r at $10\frac{1}{2}$ days (Table I). An obvious increase in the percentage of abnormal mice is noted with increasing levels of irradiation, a dose of 320r causing all progeny to be abnormal to some degree. It was observed also that whereas none of the mice treated with 80r had more than one abnormality, and only about 15 per cent treated with 160r had more than one abnormality, all of the progeny exposed to 320r had multiple malformations.

Digital abnormalities were restricted to 160 and 320r. The overall incidence of overgrowth remained approximately the same with both doses. However, a distinct difference in response between the forefeet and hindfeet was shown. Only a single case of overgrowth was found in the forefeet, while all other cases of overgrowth were observed in hindfeet.

TABLE II

Means and standard errors of litter sizes at birth after in utero *radiation*

Dose	Embryological age	Number litters	Number young	Mean ± standard error
0r		13	101	7.8 ± .6
20r	6½ days	12	92	7.7 ± .4
	10½ days	14	106	7.6 ± .7
	14½ days	13	116	8.9 ± .7
	17½ days	16	134	8.4 ± .5
80r	6½ days	14	114	8.1 ± .3
	10½ days	18	148	8.2 ± .5
	14½ days	14	122	8.7 ± .5
	17½ days	11	88	8.0 ± .7
160r	6½ days	14	97	6.9 ± .6
	10½ days	10	71	7.1 ± 1.0
	14½ days	20	167	8.4 ± .5
	17½ days	14	113	8.1 ± .6
320r	10½ days	13	60	4.6 ± .5
	14½ days	9	71	7.9 ± .5
	17½ days	24	197	8.2 ± .4

The incidence of reduction was slight after a dose of 160r, but increased to 66 per cent after 320r. The difference in response between fore- and hindfeet was not significant.

It was of primary interest to compare the response of the two broad types of genetic constitutions used in these studies: inbreds and hybrids. The comparison of the responses to the induction of developmental abnormalities is most revealing with the dose of 160r, since with 320r all progeny were affected, and with 80r, the percentage of abnormal mice was too small to detect a significant difference. Following a prenatal exposure to 160r, 64 per cent of the inbred progeny showed some malformation, but only 29 per cent of the hybrid progeny did ($X^2 = 6.10$; $0.01 < P < 0.05$).

Prenatal and neonatal mortality

Although interest in the present studies was directed primarily towards studying effects of prenatal irradiation on postnatal viability, certain conclusions may be drawn concerning effects on prenatal and neonatal viability. One measure of the effects on prenatal viability is the number of progeny that survive to term (Table II). The only treatment that differs significantly from the controls is 320r at 10½ days which caused a loss of 41 per cent of the young. All of the progeny born after treatment with 320r at 10½ days were grossly abnormal morphologically, and, in addition, were all dead by the time of recording of the birth of the litter. Partial remains of mice that had been eaten by the dam also were evident. The decreased litter size, thus, may not have been due to prenatal death

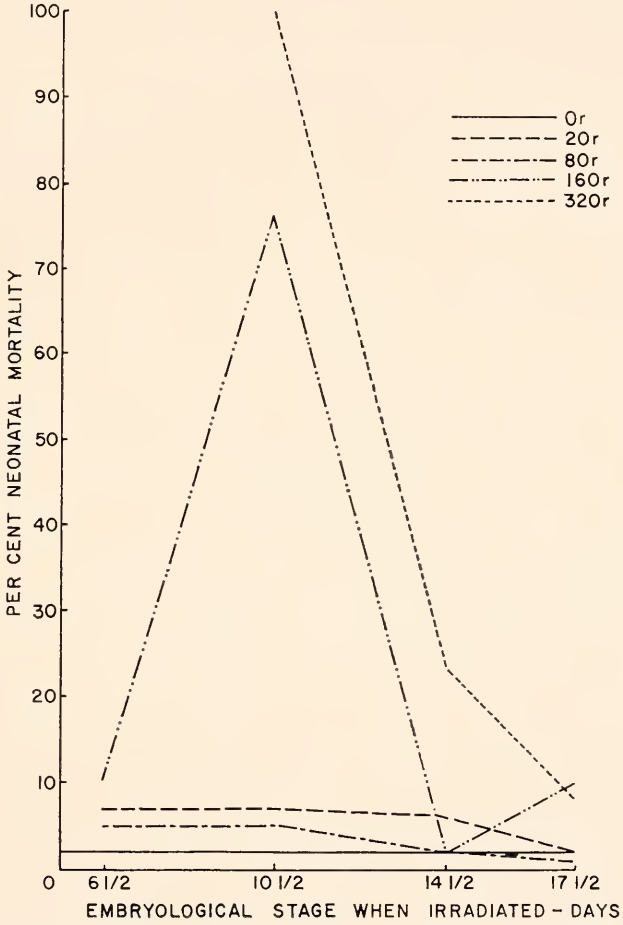

FIGURE 1. Percentage incidence of stillborn mice, all genotypes pooled, following *in vitro* irradiation.

and resorption, but may have been due to the eating of abnormal young by the dam. An additional number of mice were given 320r at 10½ days and sacrificed at 17½ days of gestation to explore the loss of individuals. It was found that of 68 embryos from 9 litters, all but two of them were already dead at the time of observation. Of the dead embryos, half of them were dead soon after treatment as evidenced by the almost complete resorption and the lack of distinguishable features of the embryos. In one of the litters all of the embryos were almost completely resorbed indicating that 320r given on 10½ days may cause a 100 per cent prenatal loss of progeny.

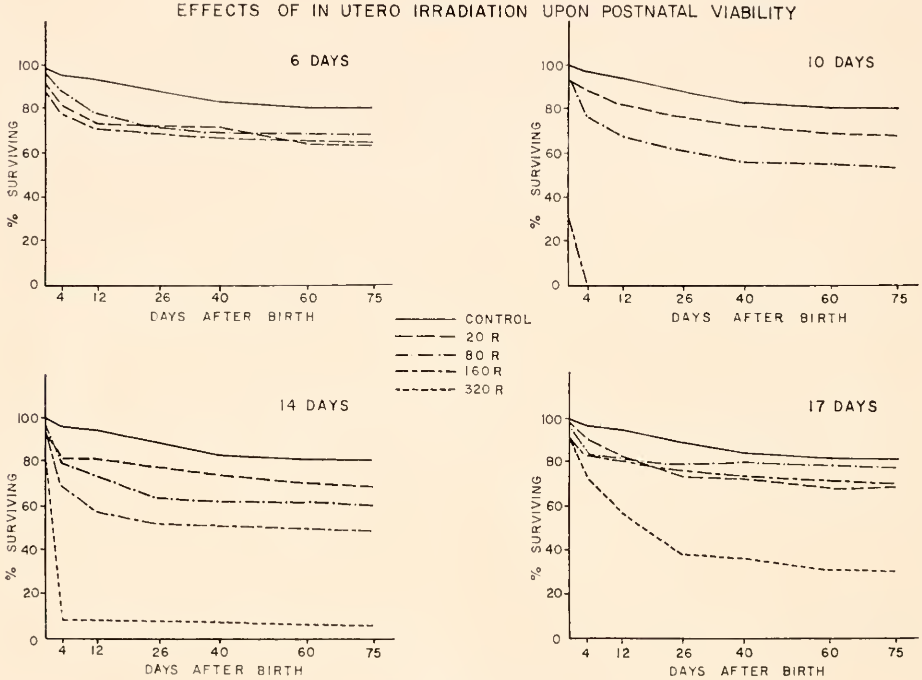

Figure 2. Effects of prenatal irradiation on postnatal survival of mice.

The percentage of progeny that were stillborn or died within 12 hours of birth reached a maximum of 100 per cent on day 10½ after a dose of 320r (Figure 1). The embryological age of 10½ days appears to be most sensitive to neonatal mortality. The LD$_{50}$ at birth is somewhere between 80 and 160r, the former causing only 5 per cent neonatal deaths, the latter 76 per cent. The only other treatment that increased neonatal mortality appreciably was 320r at 14½ days, which caused 23 per cent of the progeny to be stillborn. Doses of 20r or 80r appeared to be ineffective in increasing neonatal mortality when given at any gestational age.

A differential response of inbred and hybrid progeny was evident following a dose of 160r at 10½ days. Mortality was 100 per cent in the inbreds, but only

TABLE III

Effects of sex upon postnatal viability following prenatal irradiation
(per cent alive)

Embryonic age at treatment	Irradiation dose		Days after birth				
			4	12	26	40	60
Untreated	0r	Male	98	98	93	93	91
		Female	98	95	87	78	76
6½ days	20r	Male	95	90	88	88	85
		Female	80	70	70	67	63*
	80r	Male	95	82	74	68	67
		Female	89	82	76	76	76
	160r	Male	93	86	86	86	84
		Female	81	72	67*	63*	60**
10½ days	20r	Male	98	91	89	82	76
		Female	89*	80*	75*	73	73
	80r	Male	87	79	74	66	66
		Female	75	62	56	52	52
14½ days	20r	Male	90	90	86	84	80
		Female	84	84	79	74	69
	80r	Male	87	83	67	65	65
		Female	78	70	63	63	63
	160r	Male	83	72	64	63	61
		Female	60*	49**	44**	44**	43**
17½ days	20r	Male	92	86	77	74	71
		Female	91	82	72	72	71
	80r	Male	85	83	79	79	77
		Female	85	83	83	83	83
	160r	Male	98	95	89	86	84
		Female	88	84	81	76	74
	320r	Male	84	66	47	43	40
		Female	74*	53**	36*	35	28*

* $P < 0.05$.
** $P < 0.01$.

64 per cent in the hybrids ($X^2 = 8.83$; $0.001 < P < 0.01$). The difference between inbreds and hybrids in all other treatments was not significant. However, after 320r at 14½ days, which did cause an increase in mortality, the data support the contention that the inbreds were more sensitive than the hybrids (30 per cent and 20 per cent mortality, respectively).

TABLE IV

Comparison of postnatal viability of inbred and hybrid mice following prenatal irradiation (per cent alive)

Embryonic age at treatment	Irradiation dose		Days after birth				
			4	12	26	40	60
Untreated	0r	Inbred	87	87	83	83	83
		Hybrid	99*	96	90	83	81
6½ days	20r	Inbred	52	52	52	52	45
		Hybrid	98**	86**	85**	83**	81**
	80r	Inbred	84	84	77	74	74
		Hybrid	88	77	70	68	67
	160r	Inbred	84	78	75	75	72
		Hybrid	75	68	66	63	62
10½ days	20r	Inbred	74	72	72	67	62
		Hybrid	96**	87	79	75	73
	80r	Inbred	60	49	49	40	39
		Hybrid	93**	84**	74**	71**	71**
14½ days	20r	Inbred	76	76	66	66	63
		Hybrid	85	85	83*	78	73
	80r	Inbred	60	51	38	38	38
		Hybrid	94**	91**	83**	81**	81**
	160r	Inbred	49	40	31	30	30
		Hybrid	88**	76**	73**	73**	70**
17½ days	20r	Inbred	85	70	66	64	61
		Hybrid	93	92**	79*	78**	77*
	80r	Inbred	92	92	92	92	88
		Hybrid	81	78	75	75	75
	160r	Inbred	79	79	74	72	72
		Hybrid	85	81	77	73	70
	320r	Inbred	66	49	27	19	19
		Hybrid	78	61	47*	45**	37

Significant difference between inbred and hybrid mice.
* $P < 0.05$.
** $P < 0.01$.

Postnatal viability

Effects of prenatal irradiation on postnatal survival through 60 days of age are shown in Figure 2. The data include only those mice that survived the first 12 hours after birth. Due to prenatal and neonatal mortality, all levels of irradiation are not represented at all embryological ages. The data are presented within each of the four embryological ages pooled by sex and genotype. There appears to be some effect on postnatal viability following prenatal irradiation at any of the four gestational ages. Effects are minimum following irradiation at 6½ days, reach their maximum following exposure at 10½ days, and then decline at 14½ and 17½ days. After a dose of 80r at 10½ days only 55 per cent of the mice that were born survived until 60 days of age. Sixty-two per cent survived the same dose at 14½ days and 78 per cent at 17½ days.

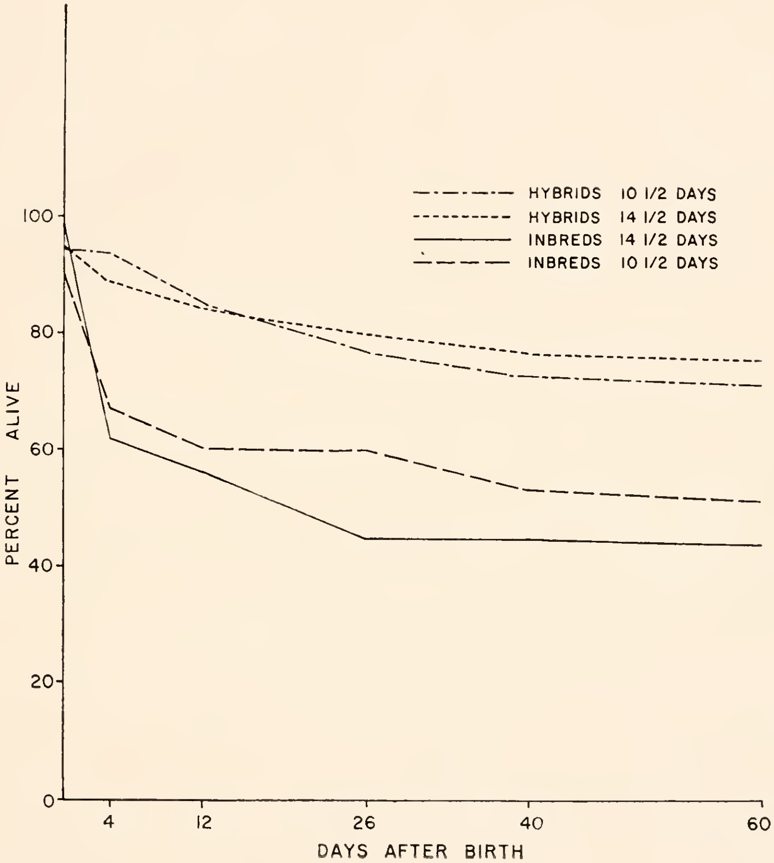

FIGURE 3. Effects of prenatal irradiation on postnatal survival of inbred and hybrid mice following irradiation at 10½ or 14½ days of gestation.

Table III gives the data on the postnatal survival of mice according to sex. The data were analyzed by means of variance analyses within each of the embryological age—irradiation dose treatment combinations. The mean squares of within sexes variances were tested against the mean squares of between sexes variances, and significant F values are indicated in Table III. In all treatments in which there is a significant difference in postnatal viability between the sexes, more female progeny die than male progeny. Among the untreated, control mice, although the difference does not reach significance, more males survive than females. There does not appear to be any consistent pattern between survival differences between the sexes and level of irradiation and gestational age at treatment. Prenatal irradiation does not appear to affect the basic sex difference in postnatal viability that was observed in these data.

The genetic data of the present investigations were analyzed by comparing the response of inbred and hybrid progeny within each of the treatments (Table IV). Among the control mice inbred progeny suffered a greater mortality than the hybrids the first few days after birth, but had comparable survival thereafter. In all of the prenatally-irradiated groups in which there was a significant difference in postnatal viability between inbred and hybrid mice, it was the inbreds that had the greater mortality. In those treatments which produced significant differences in postnatal viability, the major portion of the mortality had taken place by 12 days after birth. The greatest differences in mortality between inbreds and hybrids are exhibited following irradiation at 10½ days or 14½ days. Results of the overall response of the two types of genotypes pooled by level of irradiation are shown in Figure 2.

Discussion

Results of the present study, as well as the previous reports describing other postnatal responses of the same group of experimental mice (Nash and Gowen, 1962, 1965; Nash, 1969), have demonstrated that prenatal irradiation may have pronounced, long-term effects on postnatal morphology and function. Such effects may develop in animals that appear normal at birth. The viability findings reported in the present paper are in basic agreement with those obtained by Rugh and Wohlfromm (1965) in the mouse and Sikov, Resta, and Lofstrom (1969), in the rat. Rugh and Wohlfromm's comprehensive study indicated that the postnatal $LD_{50/30}$ values following prenatal irradiation between days 6 and 18 of gestation fluctuated with respect to certain developmental stages. They found that the most radiosensitive stages were on days 8 to 10 of gestation, a time of active differentiation of the nervous system. The methodology employed in the present studies differed from that used by Rugh and Wohlfromm (1965) in two major aspects. First, their studies utilized just one inbred strain of mice (CF_1), whereas the present studies used mice of nine different inheritance types. Secondly, in their studies all newborn litters were given to foster mothers that had not been irradiated. In the present studies litters were maintained with their natural mothers who also had been exposed to the same amount of whole-body irradiation as had their litters. Results thus reflect a combination of direct effects of radiation on the progeny and indirect effects that may have been mediated through the somatic effects of irradiation on the mother. In a previous paper (Nash and Gowen, 1962), it was pointed out that there was some indirect evidence that effects of prenatal irradiation on postnatal growth were due to direct effects on the irradiated embryos and not due to effects on the lactating ability or maternal behavior of the mother. Similar postnatal growth patterns were obtained following a dose of

320r at 17½ days gestation to fetuses and mother and a dose of 320r on the day of birth to the litter only. In addition, many of the effects on postnatal growth did not become evident until sometime after weaning. It is possible that some specific dose-embryological age combination might have an effect on the maternal organism which would influence postnatal viability of the progeny with or without a corresponding effect on postnatal growth.

With the above limitations on consideration of results of both investigations in mind, some striking quantitative differences in response to prenatal irradiation of different genotypes become evident. In general the $LD_{50/30}$ values obtained by Rugh and Wohlfromm from the CF_1 strain are higher than those of mice in the present studies, both inbreds and hybrids. The hybrids did tend to exhibit a greater radioresistance than the inbreds as measured by effects on development and postnatal viability. Heterosis in radiation response to prenatal exposure also has been noted by Rugh (1958) and Nash (1969). The present experiments do not yield any direct information on the underlying physiological mechanism of the greater radiation resistance of the hybrids. It is possible that some of the differences in response are due, in part, to differences in developmental rates which may be under genotypic control. The same chronological age of gestation need not reflect necessarily the same developmental stage of the embryos. Painter (1928) and Venge (1950) demonstrated the influence of genetically determined variation in the timing of embryological development upon subsequent growth and development. Examination of differences in size of two breeds of rabbits revealed that the differences could be traced back to early embryological stages and differences in cleavage rates of fertilized ova between large- and small-sized breeds. Additional experiments which would allow for differences in developmental rate of different genotypes are necessary. To date, this approach apparently has been used only by Dagg (1963). Differences in embryonic response also may reflect inherent differences in susceptibilities to radiation-induced effects on prenatal development and postnatal growth and viability.

Summary

1. Developmental malformations and effects on postnatal viability were measured in inbred and hybrid mice that had been irradiated at different stages of embryological development. Three genetically differentiated inbred strains of mice, BALB, K, and S, and their six possible types of hybrids, including reciprocals, were used. The design for these studies was factorial. There were five levels of irradiation: 0, 20, 80, 160, and 320r; five embryological stages: untreated, 6½, 10½, 14½, and 17½ days of gestation; two sexes; and mice of nine different inheritance types. Pregnant females were exposed to single whole-body 250 pkv x-rays on a specified day of gestation as timed from the appearance of a vaginal plug. Malformations were scored at birth and postnatal mortality was followed through 75 days of age.

2. Morphological anomalies observable at birth were found only after irradiation at 10½ days gestation. Anomalies were observed after doses of 80r or more. Neonatal mortality also was highest following irradiation at 10½ days. The LD_{50} at birth was found to be between 80 and 160r, with a dose of 320r causing all progeny to be stillborn.

3. A differential response both in the induction of malformations and in the incidence of neonatal deaths was found between inbred and hybrid genotypes. Following treatment with 160r at 10½ days there was 64 per cent abnormal and 100 per cent stillborn progeny among the inbreds compared to 29 per cent abnormal and 64 per cent stillborn among the hybrids.

4. Effects of prenatal irradiation on postnatal survival to 60 days were at a minimum after treatment at 6½ days gestation, reached a maximum following irradiation at 10½ days, and then declined at 14½ or 17½ days. No consistent patterns of survival differences between males and females and level of irradiation and embryological stage at the time of treatment was observed.

5. Among the control or non-irradiated mice, inbred progeny suffered a higher mortality than the hybrids especially during the first few days after birth. In those treatments which produced a significant increase in postnatal mortality, inbred genotypes responded to a greater degree than did hybrid genotypes.

LITERATURE CITED

Allen, E., and E. C. MacDowell, 1940. Variation in mouse embryos of eight days gestation. *Anat. Rec.*, **77**: 165–173.

Bagg, H. J., 1922. Disturbances in mammalian development produced by radium emanation. *Amer. J. Anat.*, **30**: 133–161.

Brill, A. B., W. E. Nance, E. Engel, S. R. Glasser and E. H. Forgotson, 1967. Radiation induced abnormalities in human development. Pages 69–87 in S. L. Marcus and C. C. Marcus, Eds., *Advances in Obstetrics and Gynecology, Volume 1.* Williams and Wilkins, Baltimore, Maryland.

Callas, G., 1962. Embryology of X-ray induced cleft palate in mice. *Anat. Rec.*, **142**: 336.

Dagg, C. P., 1963. Some effects of X-irradiation on the development of inbred and hybrid mouse embryos. Pages 91–102 in W. D. Carlson and F. X. Gassner, Eds., *Effects of Ionizing Radiation on the Reproductive System.* Pergamon Press, New York.

Gowen, J. W., Ed., 1952. *Heterosis.* Iowa State University Press, Ames, Iowa, 552 pp.

Gowen, J. W., and J. Stadler, 1956. Life spans of different strains of mice as affected by acute irradiation with 100 pkv X-rays. *J. Exp. Zool.*, **132**: 133–156.

Grahn, D., 1954a. Genetic variation in the response of mice to total body X-irradiation. I. Body weight response of six inbred strains. *J. Exp. Zool.*, **125**: 39–61.

Grahn, D., 1954b. Genetic variation in the response of mice to total body X-irradiation. II. Organ weight response of six inbred strains. *J. Exp. Zool.*, **125**: 63–83.

Job, T. T .,G. J. Leibold and H. A. Fitzmaurice, 1935. Biological effects of roentgen rays. Development of critical periods in mammalian development with X-rays. *Amer. J. Anat.*, **56**: 97–117.

Murphree, R. L., and H. B. Pace, 1960. The effects of prenatal radiation on postnatal development in rats. *Radiat. Res.*, **12**: 495–504.

Nash, D. J., 1969. Lifetime reproductive performance of mice exposed as embryos to X-irradiation. *Biol. Bull.*, **137**: 189–201.

Nash, D. J., and J. W. Gowen, 1961. Reproductive performance of mice exposed to embryonic X-irradiation. *Amer. Zool.*, **1**: 377.

Nash, D. J., and J. W. Gowen, 1962. Effects of X-irradiation on postnatal growth in the mouse. *Biol. Bull.*, **122**: 115–136.

Nash, D. J., and J. W. Gowen, 1963a. Lifespans of mice exposed to embryonic X-irradiation. *Rec. Genet. Soc. Amer.*, **48**: 903–904.

Nash, D. J., and J. W. Gowen, 1963b. Heterosis in the response of mouse embryos to irradiation. *Proc. XI Int. Congr. Genet., (The Netherlands)*, **1**: 78–79.

Nash, D. J., and J. W. Gowen, 1965. Effects of X-irradiation of mice exposed in utero during different stages of embryological development on duration of mature life. *Biol. Bull.*, **128**: 425–458.

O'Brien, J. P., 1956. Vertebrate radiobiology: embryology. *Annu. Rev. Nuclear Sci.,* **6**: 423–489.

Painter, T. S., 1928. Cell size and body size in rabbits. *J. Exp. Zool.,* **50**: 441–453.

Parish, N. R., R. L. Murphree and E. W. Hupp, 1962. Growth and sexual development of prenatally irradiated cattle. *J. Anim. Sci.,* **21**: 473–478.

Rugh, R., 1953. Vertebrate radiobiology: embryology. *Annu. Rev. Nuclear Sci.,* **3**: 271–302.

Rugh, R., 1958. Increased radioresistance through heterosis. *Science,* **127**: 144–145.

Rugh, R., 1959. Vertebrate radiobiology: embryology. *Annu. Rev. Nuclear Sci.,* **9**: 493–522.

Rugh, R., 1968. *The Mouse: Its Reproduction and Development.* Burgess Publishing Company, Minneapolis, Minnesota, 430 pp.

Rugh, R., and S. Jackson, 1958. Effect of fetal X-irradiation upon subsequent fertility of the offspring. *J. Exp. Zool.,* **138**: 209–221.

Rugh, R., and M. Wohlfromm, 1965. Prenatal X-irradiation and postnatal mortality. *Radiat. Res.,* **26**: 493–506.

Rugh, R., A. Duhamel, A. Chandler and A. Varma, 1964. Cataract development after embryonic and fetal X-irradiation. *Radiat. Res.,* **22**: 519–534.

Russell, L. B., 1950. X-ray induced developmental abnormalities in the mouse and their use in the analysis of embryological patterns. I. External and gross visceral changes. *J. Exp. Zool.,* **114**: 545–601.

Russell, L. B., S. K. Badgett and C. L. Saylors, 1960. Comparison of the effects of acute, continuous, and fractionated irradiation during embryonic development. Pages 343–359 in A. A. Buzzati—Traverso, Ed., *In Immediate and Low Level Effects of Ionizing Radiation.* Taylor and Francis Ltd., London.

Sikov, M. R., C. F. Resta and J. E. Lofstrom, 1969. The effects of prenatal X-irradiation of the rat on postnatal growth and mortality. *Radiat. Res.,* **40**: 133–148.

Stadler, J., and J. W. Gowen, 1957a. Contributions to survival made by body cells of genetically differentiated strains of mice following X-irradiations. *Biol. Bull.,* **112**: 400–421.

Stadler, J., and J. W. Gowen, 1957b. Radiological effects on resistance mechanisms of genetically differentiated strains of mice exposed to *Salmonella typhimurium. J. Infec. Dis.,* **100**: 284–299.

Venge, O., 1950. Studies of the maternal influence on the birth weight in rabbits. *Acta. Zool.,* **31**: 1–148.

Reference: *Biol. Bull.*, **140**: 242–254. (April, 1971)

UPTAKE AND ASSIMILATION OF AMINO ACIDS BY *PLATYMONAS*. II. INCREASED UPTAKE IN NITROGEN–DEFICIENT CELLS[1]

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Nitrate, ammonia, and atmospheric nitrogen are usually considered the only significant nitrogen sources for marine phytoplankters (Dugdale and Goering, 1967). However, organic nitrogen as free amino acid is present in nearshore and estuarine waters at concentrations comparable to those of nitrate and ammonia. Five $\times 10^{-7}$ to 5×10^{-6} moles/liter of total dissolved amino acids have been reported by several investigators (Chau and Riley, 1966; M. E. Clark, California State College at San Diego, personal communication; Degens, 1968; Hobbie, Crawford and Webb, 1968; Webb and Wood, 1967).

North and Stephens (1967, 1969) have studied the uptake and assimilation of glycine by *Platymonas*, a rock pool phytoplankter which is occasionally abundant in inshore locations. This organism grows rapidly in batch culture with glycine, nitrate, or ammonia as its sole nitrogen source (supplied at 2×10^{-3} moles/liter). Cells harvested from such cultures are capable of rapid and continuous uptake of glycine-C^{14} present at low, natural concentrations (1×10^{-6} moles/liter). Uptake rates are proportional to concentration in this concentration range. The labelled amino acid in the cells enters oxidative and synthetic pathways. The contribution of amino acid nitrogen accumulated from low ambient concentration to the nitrogen requirement of the cells can be evaluated from measurements of growth rate, amino acid uptake velocity, and cell nitrogen.

This report describes the stimulation of amino acid uptake in *Platymonas* grown on restricted nitrogen supply. The possibility of interactions between amino acid uptake and the acquisition of other nitrogenous compounds in the environment is also discussed. For example, nitrate reductase formation is suppressed by ambient ammonia in some algae (Epply, Coatsworth, and Solórzano, 1969). Conversely, amino acids are known to suppress and desuppress nitrate reductase synthesis in some higher plants (Filner, 1966). Finally, data concerning uptake of dicarboxylic and polybasic amino acids and their suitability as a nitrogen source are presented.

MATERIALS AND METHODS

The original culture of *Platymonas*, the organism employed, was obtained from H. A. Lowenstam (California Institute of Technology). The culture medium contained 2×10^{-3} or 2×10^{-4} g-at N/liter (as nitrate, glycine, glutamate, or arginine), 2×10^{-4} moles/liter K_2HPO_4, 10^{-5} moles/liter $FeCl_3$, and 0.001% EDTA in artificial seawater (Instant Ocean, Aquarium Systems Inc., Ohio). Sterile liquid cultures were inoculated from axenic agar slants. Cultures were maintained at 20° C with cool-white fluorescent lights (10^4 ergs/cm^2/sec). Con-

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tinuous culture apparatus as described in North and Stephens (1969) was also used for one series of experiments.

Cells grown on 2×10^{-3} g-at N/liter ("high" nitrogen cultures), were harvested in late log phase for uptake experiments. Cells supplied with 2×10^{-4} g-at N/liter ("low" nitrogen cultures) were harvested in late log or early stationary phase of the growth curve. It should be emphasized that the difference between high and low nitrogen cultures is qualitative in this work, and the initial concentration of nitrogen in both cultures is considerably higher than levels in the sea.

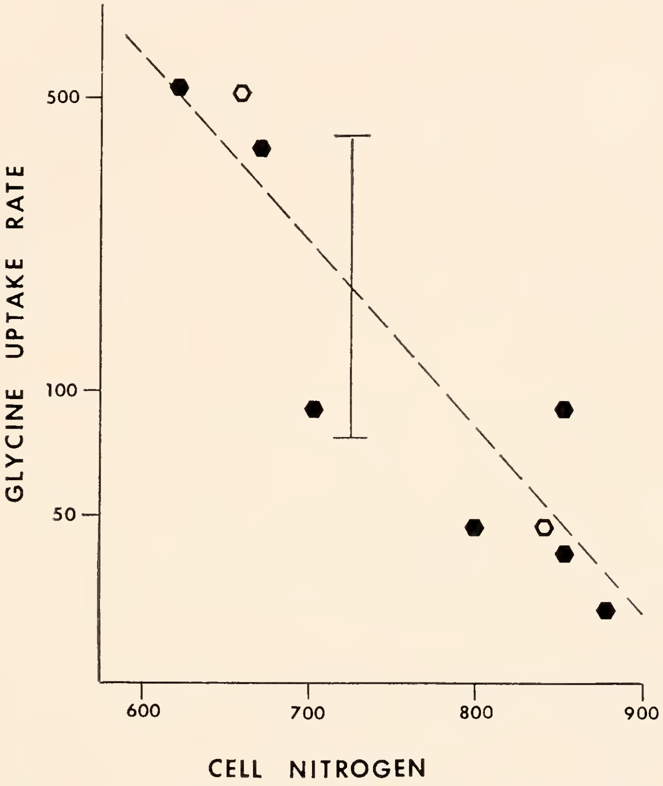

FIGURE 1. Glycine uptake *vs.* cell nitrogen content for *Platymonas* from continuous culture. Nitrogen content varied in response to changes in dilution rate of the culture medium; abscissa: μg N/10^8 cells; ordinate: moles glycine $\times 10^9$/hr/10^8 cells; (○) are cells from steady-state growth conditions. The vertical bar delineates 2 standard errors on each side of the regression line.

For amino acid uptake measurements, cells were harvested, washed, and resuspended at 5×10^5 cells/ml in Millipore-filtered artificial seawater. C^{14} amino acids were added to give 1 μc/20 ml of cell suspension. For glycine, arginine, and glutamate, this produced substrate concentrations of 2 to 4×10^{-7} moles/liter. The amino acid analogues α-amino isobutyric acid (AIB), 1-amino-1-carboxy-cyclopentane (cycloleucine), and the amino acid taurine, had lower specific activities, and minimum substrate concentrations were about 10^{-5} moles/liter (*cf.* Table II). Higher concentrations were achieved with addition of C^{12} amino acids.

To determine uptake rates, 1 ml aliquots were taken periodically. The cells in the aliquot were collected on filters (0.45 μ, EHWP, Millipore), washed with 10 ml artificial seawater, and the radioactivity in the cells counted. Counts/minute (cpm) in the first aliquot and the time of sampling (1 or 2 minutes) were treated as blank values and subtracted from all subsequent samples. Uptake rates were also measured by following the disappearance of C^{14} from the seawater. Radioactivity in 1 ml aliquots of seawater was counted after removing cells by filtration. All samples were counted in a Beckman CPM-100 liquid scintillation system, corrected for quenching and background, and cpm converted to moles. Amino acid uptake rates were obtained from short term observations during which rates remained linear with time.

Uptake velocities were calculated by the weighting method of Wilkinson (1961): for $Y = Vx$ (Y = moles taken up at time x at velocity V), the standard error (S.E.) of Y was assumed to be proportional to Y because of volumetric sampling errors. That is, S.E. $(Y) = KY$ where K is a constant per cent error in sampling. This provides a weighting factor of $1/Y^2$ for calculation of the velocity V and the S.E. of V.

Uptake velocities were determined at several substrate concentrations. From such data, North and Stephens (1967) have shown that glycine accumulation involves a saturable step and can be adequately described using the Michaelis-Menton equation, $V = V_{\max}(S/S + K_s)$ where V = uptake velocity, $V_{\max}$ = maximum uptake velocity, S = substrate concentration, K_s = substrate concentration at which the velocity is half-maximal. An unweighted V vs. V/S linear transformation of the Michaelis-Menten equation was used to evaluate the kinetic parameters K_s and $V_{\max}$ (*cf. Figure* 2). This transformation was employed in preference to the more commonly used Lineweaver-Burk plot ($1/V$ *versus* $1/S$) because it provides more accurate estimates of K_s and $V_{\max}$ (Dowd and Riggs, 1965).

Cells were extracted with cold 5% trichloroacetic acid (TCA) to provide a measure of C^{14} assimilation. This solubilizes amino acids and various small organic compounds but does not extract nucleic acids, lipids, polypeptides, or protein. In experiments with C^{14} amino acid analogues, cells were extracted with cold TCA, 80% ethanol, and 0.66 N perchloric acid. Insoluble and total radioactivity were counted directly, and soluble activity calculated by difference. Cell nitrogen content was measured with a semi-micro Kjeldahl technique (Steyermark, 1961) in continuous culture experiments and with a Coleman nitrogen analyzer for other experiments.

RESULTS

Amino acid uptake velocities increased when nitrogen supply in the culture medium was lowered. This stimulation occurred in cells harvested from both batch and continuous culture. A logarithmic increase in rate of glycine uptake was correlated with a decrease in nitrogen content in populations from continuous culture (Fig. 1). A similar rate increase occurred in batch culture cells (Table I).

TABLE I

Amino acid uptake by Platymonas *grown on two concentrations of nitrate, glycine, arginine, and glutamate*

Expt	Culture conditions			Amino acid uptake				Nitrogen accumulation	
	Nitrogen source	Nitrogen concentration*	Cell number $\left(\frac{\text{cells} \times 10^{-5}}{\text{ml}}\right)$	C^{14} amino acid	Amino acid concentration (moles/liter)	Uptake rate $\left(\frac{\text{moles} \times 10^{9}}{\text{hr } 10^{8} \text{ cells}}\right)$	Uptake rate† $\left(\frac{\mu\text{g N}}{\text{hr } 10^{8} \text{ cells}}\right)$	N content $\left(\frac{\mu\text{g N}}{10^{8} \text{ cells}}\right)$	Gen. Time†† (hours)
1	NO_3	low	7.9	glycine	3.6×10^{-7}	128	2.42	562	160
	NO_3	high	9.1	glycine	3.6×10^{-7}	32	0.60	870	1005
	GLY	low	15.2	glycine	3.6×10^{-7}	60	1.13	382	234
	GLY	high	14.8	glycine	3.6×10^{-7}	4	0.07	838	8290
2	NO_3	low	9.3	glycine	4.2×10^{-7}	152	2.54	379	103
	NO_3	high	20.7	glycine	4.2×10^{-7}	39	0.64	474	514
	ARG	low	8.8	glycine	4.2×10^{-7}	215	3.43	563	114
	ARG	high	26.2	glycine	4.2×10^{-7}	43	0.72	815	785
	GLU	low	9.0	glycine	4.2×10^{-7}	183	3.06	473	107
	GLU	high	40.8	glycine	4.2×10^{-7}	99	1.65	513	215
	NO_3	low	9.3	arginine	1.5×10^{-7}	81	15.12	379	17
	NO_3	high	20.7	arginine	1.5×10^{-7}	33	6.16	474	53
	ARG	low	8.8	arginine	1.5×10^{-7}	83	15.51	563	25
	ARG	high	26.2	arginine	1.5×10^{-7}	32	5.98	815	94
	NO_3	low	9.3	glutamate	2.5×10^{-7}	7	0.18	379	1460
	NO_3	high	20.7	glutamate	2.5×10^{-7}	1	0.02	474	19300
	GLU	low	9.0	glutamate	2.5×10^{-7}	13	0.37	473	886
	GLU	high	40.8	glutamate	2.5×10^{-7}	1	0.04	513	9120

* Low = 2×10^{-4} g-at N/liter; high = 2×10^{-3} g-at N/liter.

† Uptake rate at a substrate concentration of 5×10^{-7} moles/liter, calculated from measured rates assuming that rate is proportional to substrate concentration.

†† Generation time for a population whose growth is limited by amino acid nitrogen uptake from 5×10^{-7} moles/liter:

$$\text{Generation time} = \frac{\ln 2 \times \text{N content}}{\text{Uptake rate}}.$$

The low nitrogen cells (*i.e.*, cells grown on 2×10^{-4} g-at N/liter) contained less cell nitrogen and acquired amino acids from solution more rapidly than high nitrogen cells (grown on 2×10^{-3} g-at N/liter). Stimulation also appeared to be independent of the form in which the nitrogen was supplied during growth. Four different nitrogen sources were employed (nitrate, arginine, glutamate, and glycine) each at high and low concentrations. Division rates in log phase were comparable on all substrates and at both nitrogen concentrations.

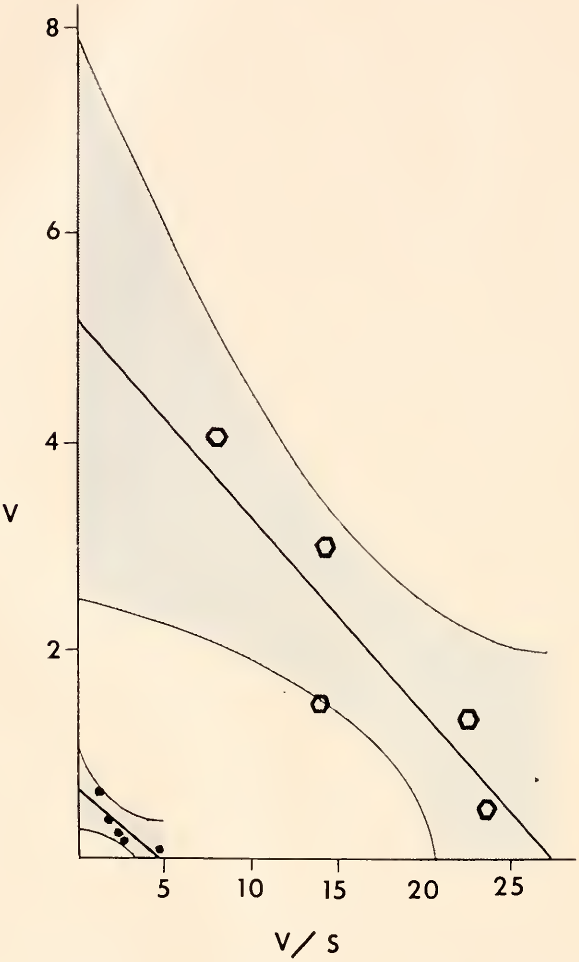

FIGURE 2. The relation between glycine uptake rates and glycine concentration for cells grown on 2×10^{-3} and 2×10^{-4} M NO_3. The plot is a linear transformation of the Michaelis-Menten equation: $V = K_s(V/S) + V_{max}$; abscissa: V/S where V = moles glycine $\times 10^6$/hr/10^8 cells and S = moles glycine $\times 10^7$/liter; ordinate: V = moles glycine $\times 10^6$/hr/10^8 cells. The solid lines were determined by a least squares analysis. The dotted line encloses the 95% confidence band.

Cells from each culture were checked for their ability to acquire arginine, glycine, and glutamate from dilute solution. The resulting uptake patterns were comparable in each high and low nitrogen pair (Table I). In every case, rates from low nitrogen cultures were substantially higher than corresponding high nitrogen cells. In addition, arginine uptake always exceeded glycine, and glutamate rates were always lowest. This pattern was independent of the nitrogen source on which the cells had been grown. Even though low nitrogen conditions consistently increased amino acid uptake rates and decreased cell nitrogen, the overall correlation between nitrogen content and uptake rate found in continuous culture (Figure 1) was not found in these batch culture experiments.

In the first experiment summarized in Table I, cells were harvested in log phase growth when cell densities and growth rates were comparable in both high and low nitrogen cultures. Therefore, nitrogen was not growth-limiting in this experiment. In the second experiment, the low nitrogen cultures were in the early stationary phase, were less dense than the high nitrogen cultures, and presumably had exhausted the available nitrogen. Nonetheless, amino acid uptake was stimulated in both experiments.

TABLE II

Uptake of amino acids and analogues by Platymonas *grown on two concentrations of nitrate*

Culture conditions		Analogue uptake				Accumulation ratios **
$[NO_3]$ (moles/liter)	[Cell] (cells/ml)	C^{14} analogue	Concentration (moles/liter)	Uptake rate $\left(\frac{\text{moles} \times 10^9}{\text{hr } 10^8 \text{ cells}}\right)$	Uptake* rate	$\left(\frac{\text{moles/liter inside}}{\text{moles/liter outside}}\right)$
2×10^{-4}	5.9×10^5	GLYCINE	4.7×10^{-7}	194	4128	—
2×10^{-4}	5.9×10^5	AIB	114×10^{-7}	372	326	254
2×10^{-4}	5.9×10^5	CYCLOLEUCINE	127×10^{-7}	112	88	65
2×10^{-4}	5.9×10^5	TAURINE	218×10^{-7}	—	—	—
2×10^{-3}	16.8×10^5	GLYCINE	4.7×10^{-7}	25	532	—
2×10^{-3}	16.8×10^5	AIB	114×10^{-7}	26	23	18
2×10^{-3}	16.8×10^5	CYCLOLEUCINE	127×10^{-7}	—	—	—
2×10^{-3}	16.8×10^5	TAURINE	218×10^{-7}	—	—	—

* Uptake rate at a substrate concentration of 10^{-5} moles/liter, calculated from measured rates assuming that rate is proportional to substrate concentration.

** Inside concentration calculated using packed cell volume (70×10^{-14} liters H_2O/cell).

The maximum velocity of glycine uptake ($V_{\max}$) and the concentration at which the system mediating uptake was half-saturated (K_s) were evaluated for high and low nitrogen cells. Glycine uptake rates were measured at ambient concentrations ranging from 2×10^{-6} to 5×10^{-5} moles/liter for low and high nitrogen cultures (Figure 2). K_s and $V_{\max}$ were evaluated from the slope and intercept of the line. In cells from low nitrogen cultures, $V_{\max} = 5181 \pm 2677$ ([moles glycine $\times 10^{-9}$/hr 10^8 cells] $\pm 95\%$ confidence interval), $K_s = 19 \pm 15$ ([moles glycine $\times 10^{-6}$/liter] $\pm 95\%$ confidence interval). In high nitrogen cultures, $V_{\max} = 644 \pm 381$, $K_s = 14 \pm 14$. A portion of the increase in $V_{\max}$ may be due to the larger size of cells from the low nitrogen culture. These cells may have 50% more surface area than high nitrogen cells. However, this explanation accounts for only a fraction of the difference observed in V_{max}.

Thus, amino acids were acquired from the medium more rapidly by cells grown on restricted nitrogen, whether in continuous culture or in fixed volume cultures. It was possible that stimulation of uptake resulted from more rapid utilization of amino acid within the cells (*i.e.*, assimilation), particularly since glycine is rapidly converted into an array of alcohol-soluble compounds (North and Stephens, 1967). This could stimulate uptake rates without enhancing the ability of the cell to accumulate amino acids against an internal concentration gradient. Accumulation of C^{14} taurine and C^{14} amino acid analogues by cells grown in high nitrogen and low nitrogen batch cultures was compared. Taurine was not acquired by the cell under any of the conditions employed. Alpha-aminoisobutyric acid (AIB) and 1-amino-1-carboxy-cyclopentane (cycloleucine), however, are accumulated much more rapidly by nitrogen deficient cells (Table II). AIB-C^{14} and cycloleucine-C^{14} in the cells were readily extracted with 80% ethanol, verifying that these analogues were not metabolized into insoluble compounds. In another experiment, AIB was extracted quantitatively from *Platymonas* and identified by autoradiography of a thin layer chromatogram of an extract (perchloric acid). The ratio of internal to external concentration of these analogues was high at the end of the experiment (Table II) and indicated accumulation against a substantial concentration differ-

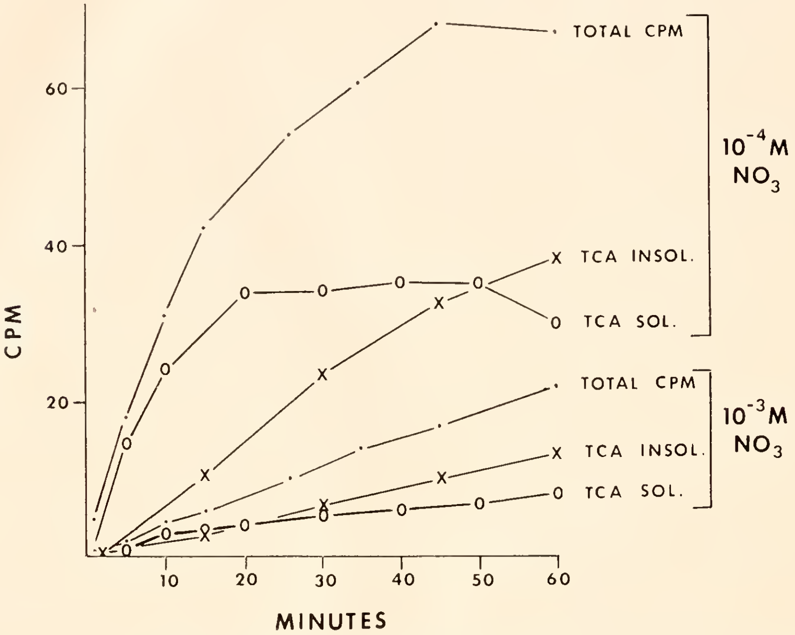

FIGURE 3. Incorporation of glycine-C^{14} into TCA-soluble and TCA-insoluble fractions of *Platymonas* grown on 2×10^{-3} and 2×10^{-4} M NO_3; (•——•) represents total CPM in cells; (X——X) CPM in TCA-insoluble fraction; (O——O) CPM in TCA-soluble fraction.

ence. Larger ratios would have been achieved had the experiment continued since uptake was still linear at the end of the observations. Thus, uptake stimulation is accompanied by increased rates of accumulation into the cell.

The rate at which glycine radioactivity was assimilated into the cold TCA-insoluble fratcion of cells from low and high nitrogen cultures was also investigated. The amino acid C^{14} obtained from the medium entered synthetic pathways more rapidly in the low nitrogen cells (Fig. 3). No conclusions can be drawn about absolute rates of amino acid assimilation without further information about the size and specific activities of amino acid pools. However, the data indicate that ambient amino acids were incorporated into cells of either history.

The rates of arginine uptake were unaffected by the presence of nitrate or ammonia at levels 20 to 1000 times more concentrated than the amino acids (Table III). A similar result was obtained with glycine-C^{14}. Thus, there is no short term interaction at levels of inorganic nitrogen as high as 10^{-4} moles/liter. Of course, our earlier results indicate that supplying nitrate at levels around 2×10^{-3} moles/liter would eventually depress amino acid uptake rates, but the time course of this effect has not been investigated. However, such levels far exceed any natural concentrations that might be encountered by *Platymonas*.

TABLE III

Arginine uptake by Platymonas *in the presence of nitrate and ammonia. Cells were incubated in* 1.5×10^{-7} M *arginine-*C^{14} *for 10 minutes*

	% radioactivity remaining in medium at 10 minutes	
	High nitrogen culture	Low nitrogen culture
control	75.2	28.6
10^{-5} *M* NO_3	76.5	28.7
10^{-4} *M* NO_3	75.5	29.4
10^{-5} *M* NH_3	75.9	28.1
10^{-4} *M* NH_3	78.2	28.7

DISCUSSION

We are investigating the significance of natural dissolved amino acids to the nutrition of *Platymonas*. The fact that arginine, glutamate, and glycine can provide nitrogen for growth in batch culture is neither necessary nor sufficient to argue for a normal nutritional role of dissolved amino acids. An amino acid or other organic compound in the environment may supplement other nitrogen sources or compensate for a particular synthetic deficiency without being a complete nitrogen source. In addition, an amino acid that supports growth at high concentrations may have no importance in the sea. Nonetheless, neutral, dicarboxylic, and polybasic amino acids are all complete nitrogen sources for *Platymonas*. In general, utilization of amino acids supplied at the high concentrations characteristic of batch cultures appears to be widespread among phytoplankters (Danforth, 1962).

A more cogent approach to the nutritional role of dissolved amino acids is to study the utilization of these compounds when supplied at concentrations reported from natural waters. The work reported here and in earlier contributions from our laboratory supports the following statements: (1.) Amino acids are accumulated by *Platymonas* from very dilute solutions, concentrations of which are in the range reported for natural waters. (2.) Accumulated amino acids enter respiratory and synthetic pathways in the cell. (3.) Uptake continues for long periods (demonstrated for glycine). (4.) The uptake rate depends on the amino acid and the nutritional history of the cells. Rates are higher in cells which have been grown under conditions where nitrogen is restricted though not to the extent that it limits the growth rate. (5.) When cells are stimulated to take up amino acids more rapidly, the pattern of uptake is constant regardless of the nitrogen source on which they were grown. The uptake rate of arginine is always greater than glycine, and glutamate is consistently lowest. (6.) The stimulation of amino acid uptake is accompanied by increased rates of accumulation, as shown by accelerated uptake of amino acid analogues. (7.) Cells grown at reduced nitrogen concentrations show an increase in the maximum velocity ($V_{\max}$) of amino acid uptake. No change in K_s is apparent. (8.) Amino acid uptake proceeds in the presence of nitrate and ammonia.

The conditions that increase amino acid uptake in the laboratory might be expected to occur under natural conditions. We have previously shown (North and Stephens, 1969) that restriction of nutrient levels in continuous culture produced a marked increase in rates of amino acid uptake. In the present report, increased rates were also obtained when nitrogen supply was reduced in batch cultures. North, Stephens and North (in press) have also demonstrated comparable increases in uptake rates for various amino acids in several other marine phytoplankters.

There are other reports of amino acid uptake by phytoplankton. A light-dependent glycine transport system has been reported for the freshwater alga, *Scenedesmus* (Cseh and Szabo, 1965). Hellebust and Guillard (1967) report rapid uptake of glutamate by the diatom, *Melosira*. Glutamate uptake is also reported in a study of four phytoplankters by Sloan and Strickland (1965) though they conclude that the observed rates are too low to have any nutritional significance. In *Platymonas,* glutamate uptake is also quite slow compared to glycine and arginine. Even though growth on restricted nitrogen increases glutamate uptake ten-fold, rates are too low to supply a significant amount of nitrogen (see below). Culture conditions may be responsible for some of the differences found in the literature.

Other nitrogen-sensitive transport systems have been reported in algae. Yaden (1965) discusses increases in glucose and amino acid uptake in *Euglena*. Uptake was stimulated by heterotrophic growth in the dark, starvation in the dark, or streptomycin bleaching. Nitrate (Eppley and Thomas, 1969) and ammonia (Fitzgerald, 1968; Syrett, 1962) uptake systems may also respond to nitrogen deprivation. The former authors report an increase in $V_{\max}$ for nitrate uptake in nitrogen-deficient cells. Although our data justify the conclusion that $V_{\max}$ increases in batch culture cells grown under low nutrient conditions, the limitations of the batch culture technique are severe. We can neither control nor predict the growth conditions which will give reproducible values for a particular kinetic parameter in such cultures. Thus, there is no overall correlation between uptake rates and any of the usual culture parameters such as growth rate, nitrogen content, cell concentration, *etc.* This is not surprising since the conditions in batch cultures are changing continuously and rapidly.

Even though some data reported here suffer the limitations of batch culture technique, the contribution of amino acid uptake to the nitrogen economy of the cells was evaluated. Nitrogen uptake velocities (from a solution of 5×10^{-7} moles/liter) were calculated for glutamate, glycine, and arginine for cells from high and low nitrogen cultures. Uptake rates, when divided by cell nitrogen content, are equivalent to growth constants in terms of nitrogen (Dugdale and Goering, 1967); that is, the constants represent growth rates or generation times (generation time = ln 2/growth rate) assuming that growth is limited by amino acid nitrogen. The calculated constants (Table I) indicate that glycine might supply sufficient nitrogen to support growth at generations as short as 160 hours. Arginine uptake is consistently faster than glycine, and the greater nitrogen content of the molecule could support a generation as short as 17 hours. These calculated generation times cannot be compared directly with generation times under natural conditions, since the latter have not been measured. One can speculate that natural doubling times might well be ten times longer than generation times observed in batch culture. However, if calculated generation times exceed batch culture growth by fifty-fold, the substrate concerned probably cannot contribute significantly to the economy of the organism under natural conditions. Since the shortest generation time observed in batch culture of *Platymonas* was 22 hours, we conclude that glycine and arginine may be important nitrogen sources in the sea. Uptake of glutamate was slower than both arginine and glycine and could support a generation time of 886 hours. This does not represent a significant nitrogen contribution under any of our conditions (although glutamate was a competent nitrogen source in batch culture). Again the limitations of batch culture should be emphasized.

Caperon (1969) suggests that nitrogen limited cell growth should be described by a response function which incorporates both the nutritional history of the cell and the current growth conditions. Such a response function is undoubtedly required to describe behavior of the cell populations in batch culture. Until this is done, kinetic parameters obtained from such experiments should probably not be used to predict behavior of natural phytoplankton populations. This difficulty can be eliminated if cells are grown in continuous culture and allowed to reach a steady state. Under steady-state conditions, the cells have a constant and reproducible nutritional history, and well defined growth parameters.

When generation times calculated from amino acid uptake velocities and nitrogen content are compared with measured generation times of the steady-state cell populations, a more defensible estimate of the nutritional significance of dissolved amino acids is achieved (*cf.* Figure 1). At moderate growth rates (generation time = 51 hours) and natural glycine concentrations (5×10^{-7} moles/liter), the uptake system can supply sufficient nitrogen to support a calculated doubling time of 860 hours. However, at the slower growth rates achieved by reducing the nutrient supply (generation time = 166 hours), the glycine contribution becomes more important. Under these conditions, uptake rate increases and cell nitrogen decreases as in low nitrogen batch cultures. Glycine uptake could support a generation of 95 hours. This assumes that amino acid uptake continues unabated. Sustained uptake has been demonstrated for high nitrogen cells (North and Stephens, 1969), but similar experiments have not been performed on nitrogen-deficient cells.

These calculations do not correct for leakage of nitrogen-containing compounds. However, such losses are probably small. Hellebust (1965) reports that *Tetraselmis* (*Platymonas*) leaks 1–3% of its photo-assimilated carbon, depending on light conditions. If the excreted product is entirely protein, this leakage rate adds a maximum of 5% to the cell nitrogen requirement.

The amino acid molecule also provides reduced carbon to the cells. The accumulated amino acid can be expressed as a fraction of respiratory rate by calculating the oxygen required for complete oxidation of accumulated amino acid and comparing this figure to oxygen consumption in the dark. Dark respiration of *Platymonas,* as determined in a Gilson respirometer, ranges from 1.3 to 3.5 μmoles $O_2/hr/10^8$ cells. Populations from continuous cultures can obtain 0.5 μmoles glycine/hr/10^8 cells. Complete oxidation of this amount of glycine requires about 0.9 μmoles of O_2, or 25–67% of the total respiration.

This is not to say that *Platymonas* can grow exclusively on amino acid carbon in the dark, even though amino acid uptake is not light-dependent (North and Stephens, unpublished results). On the contrary, *Platymonas* appears to be an obligate phototroph (Gibor, 1956). Nonetheless, the utilization of amino acids or other organic substrates reduces the photosynthetic carbon requirements and may be important to algae in certain habitats (Bunt, 1969).

Though there is no short-term effect on amino acid uptake by high concentrations of nitrate or ammonia, we have not examined the possibility of a reciprocal situation, *i.e.,* the affect of amino acids on the utilization of other nitrogen sources, particularly nitrate. Such investigations may be important in view of recent ecological emphasis on the characteristics of nitrate uptake. Dugdale and Goering (1967) suggest using uptake velocities for nitrate and ammonia to measure nitrogen flow in primary productivity studies *in situ.* K_s and $V_{\max}$ have been used to analyze and predict interspecies competition and succession (Dugdale, 1967; Eppley, Rogers and McCarthy, 1969). Finally, a nitrate reductase assay in the field has been proposed as a sensitive measure of rates of nitrate assimilation and the presence of nitrate (Eppley, Coatsworth, and Solórzano, 1969). Interactions between nitrite, nitrate, and ammonia assimilation were examined and taken into account in the above studies. The effect of amino acids on these systems is unknown, however, and these authors have emphasized the need for such investigations. We propose to study the effect of low concentrations of amino acids on nitrate reductase content of steady-state populations.

SUMMARY

The marine phytoplankter, *Platymonas,* increases rates of amino acid uptake when grown on a restricted nitrogen supply. Uptake of glycine, arginine, and glutamate increases as much as 10-fold when cells are deprived of nitrogen during growth. In a given culture, arginine uptake is always faster than glycine, and

glutamate accumulation is always slowest. Nitrogen-deprivation also stimulates accumulation of non-metabolizable amino acid analogues. Kinetic studies suggest that V_{max} increases and K_s remains unchanged in N-deprived cells. When low concentrations of glycine or arginine are supplied to cells grown on restricted nitrogen, uptake rates are sufficient to fulfill the nitrogen requirement in *Platymonas*. High concentrations of nitrate or ammonia do not interfere with amino acid uptake. Thus amino acids in the ocean may be an important nitrogen source for some phytoplankters.

LITERATURE CITED

BUNT, J. S., 1969. Observations on photoheterotrophy in a marine diatom. *J. Phycol.*, **5**: 37–42.

CAPERON, J., 1969. Time lag in population growth response of *Isochrysis galbana* to a variable nitrate environment. *Ecology*, **50**: 188–192.

CHAU, Y. K., AND J. P. RILEY, 1966. The determination of amino-acids in seawater. *Deep Sea Res.*, **13**: 1115–1124.

CSEH, E., AND E. SZABO, 1965. The glycine uptake of *Scenedesmus obtusiusculus*. *Annal. Biol. Tihany, Hungaria*, **32**: 247–253.

DANFORTH, W. F., 1962. Substrate assimilation and heterotrophy. Pages 99–123 in R. A. Lewin, Ed., *Physiology and Biochemistry of Algae*. Academic Press, New York.

DEGENS, E. T., 1968. Molecular composition of nitrogenous compounds in seawater. *Woods Hole Oceanogr. Inst. Ref. No.* **6–52**: 1–27.

DOWD, J. E., AND D. S. RIGGS, 1965. A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. *J. Biol. Chem.*, **240**: 863–869.

DUGDALE, R. C., 1967. Nutrient limitation in the sea: dynamics, identification, and significance. *Limnol. Oceanogr.*, **12**: 685–695.

DUGDALE, R. C., AND J. J. GOERING, 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. *Limnol. Oceanogr.*, **12**: 196–206.

EPPLEY, R. W., J. L. COATSWORTH AND L. SOLÓRZANO, 1969. Studies of nitrate reductase in marine phytoplankton. *Limnol. Oceanogr.*, **14**: 194–205.

EPPLEY, R. W., J. N. ROGERS AND J. L. MCCARTHY, 1969. Half-saturation constants for uptake of nitrate and ammonium by marine phytoplankton. *Limnol. Oceanogr.*, **14**: 912–920.

EPPLEY, R. W., AND W. H. THOMAS, 1969. Comparison of half-saturation constants for growth and nitrate uptake of marine phytoplankton. *J. Phycol.*, **5**: 375–379.

FILNER, P., 1966. Regulation of nitrate reductase in cultured tobacco cells. *Biochim. Biophys. Acta*, **118**: 299–310.

FITZGERALD, G. P., 1968. Detection of limiting or surplus nitrogen in algae and aquatic weeds. *J. Phycol.*, **4**: 121–126.

GIBOR, A., 1956. The culture of brine algae. *Biol. Bull.*, **111**: 223–229.

HELLEBUST, J. A., 1965. Excretion of some organic compounds by marine phytoplankton. *Limnol. Oceanogr.*, **10**: 192–206.

HELLEBUST, J. A., AND R. R. L GUILLARD, 1967. Uptake specificity for organic substrates by the marine diatom, *Melosira nummuloides*. *J. Phycol.*, **3**: 132–136.

HOBBIE, J. E., C. CRAWFORD AND K. L. WEBB, 1968. Amino acid flux in an estuary. *Science*, **159**: 1463–1464.

NORTH, B. B., AND G. C. STEPHENS, 1967. Uptake and assimilation of amino acids by *Platymonas*. *Biol. Bull.*, **133**: 391–400.

NORTH, B. B., AND G. C. STEPHENS, 1969. Amino acids and *Platymonas* nutrition. Pages 263–273 in R. Margalef, Ed., *Proceedings of VIth Int. Seaweed Symposium*. Subsecretaria Mercante Marina, Madrid.

NORTH, W. J., G. C. STEPHENS AND B. B. NORTH, in press. Marine algae and their relations to pollution problems. *Proc. FAO Tech. Conf. on Marine Pollution*, Rome, 1970, in press.

SLOAN, P. R., AND J. D. H. STRICKLAND, 1966. Heterotrophy of four marine phytoplankters at low substrate concentrations. *J. Phycol.*, **2**: 29–32.

STEYERMARK, A., 1961. Nitrogen: micro-kjeldahl method. Pages 188–220 in *Quantitative Organic Microanalysis.* Academic Press, New York.

SYRETT, P. J., 1962. Nitrogen assimilation. Pages 171–188 in R. A. Lewin, Ed., *Physiology and Biochemistry of Algae.* Academic Press, New York.

WEBB, K. L., AND L. WOOD, 1967. Improved techniques for analysis of free amino acids in seawater. Pages 440–444 in N. B. Slova, Ed., *Automation in Analytic Chemistry. Technicon Symposium.* Mediad, New York.

WILKINSON, G. N., 1961. Statistical estimations in enzyme kinetics. *Biochem. J.*, **80**: 324–332.

YADEN, S., 1965. Uptake of organic compounds by *Euglena gracilis. Ph.D. thesis, University of Minnesota,* 33 pp.

Reference: *Biol. Bull.*, **140**: 255–273. (April, 1971)

BEHAVIORAL AND ELECTROPHYSIOLOGICAL STUDIES OF HYDRA. I. ANALYSIS OF CONTRACTION PULSE PATTERNS.[1]

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A characteristic feature of the behavior of some hydroid polyps is that their spontaneous activity consists of rhythmically re-occuring behavioral events. Examples are: the concerted oral movements of the tentacles followed by peristalsis of the proboscis in *Tubularia* (Josephson and Mackie, 1965); hydranth contraction of *Obelia* (Morin, 1969); stalk contraction in isolated polyps of *Corymorpha* (Rushforth, unpublished observations); and contraction of the body column and tentacles in *Hydra* (Passano and McCullough, 1963). In these four hydroids electrical potentials are associated with the periodic movements. The potentials are relatively large, on the order of several millivolts when externally recorded.

Large spontaneous potentials and associated periodic movements, however, are not universal in hydroids. Neither rhythmic behavior nor endogenous events have been observed in *Pennaria* (Fred Delcomyn, University of Oregon, personal communication) and *Hydractinia* (Darrell Stokes, University of Hawaii, personal communication). In *Cordylophora,* although very slow rhythmic behavior occurs (Fulton, 1963), the animal is electrically silent unless fed or stimulated (Mackie, 1968). At the present time, no hypotheses have been proposed to explain the existence of these divergent classes of hydroid behavior.

The behavior and electrical activity in Hydra

Passano and McCullough (1962) were the first investigators to record electrical correlates of spontaneous behavior in *Hydra.* In a series of papers they described the properties of two endogenously active coördinating systems in this animal (Passano and McCullough, 1964, 1965; McCullough, 1965).

One system termed the Rhythmic Potential System, produces pulses (RP's) rhythmically with frequencies ranging from 1 to 10 per minute. These pulses are small, relatively rapid, compound potentials on the order of tens of microvolts when recorded externally. While potential RP pacemakers are located throughout the whole column, RP's usually begin in the basal region of the animal. The pulses are conducted in the column at about 4 cm per sec. Such rhythmic potentials trigger gastrodermal muscle contraction, causing elongation of the body column (Shibley, 1969).

A second coördinating system produces large, slow potentials when recorded with external electrodes. The pulses are of up to 500 msecs in duration and have amplitudes up to several tens of millivolts. The system was designated the Contraction Burst System by Passano and McCullough (1964), since the pulses were associated with contraction of the epidermal musculature and occurred usually in bursts. On the basis of long-term recordings (lasting 10 to 12 hours) from five species of hydra, Rushforth (1966) found that in many animals the electrical pulses and body contractions occur primarily as single, widely spaced events rather than in bursts. In view of this finding the potentials were termed Contraction Pulses (CP's) rather than Contraction Burst Potentials. This term has been used subsequently by Rushforth (1967a) and others (Josephson, 1967, Josephson and Macklin, 1967, 1969). In retrospect, it would have been more appropriate to have called these potentials Column Contraction Pulses to distinguish them from contraction pulses recorded from the tentacles of the animal (Rushforth, 1967b). However, in order not to add another term to the rapidly proliferating nomenclature of pulses recorded from hydroids, the term Contraction Pulses (CP's) will be retained in the present study. The pulses are equivalent to those designated as Contraction Burst Potentials by Passano and his co-workers.

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The present study is a quantitative description of the temporal patterns of Contraction Pulses in two species of *Hydra:* the large Belgian species *H. pirardi* Brien and the North American variety *H. pseudoligactis.* It is likely that *H. pseudoligactis* is synonymous with *H. canadensis* which deserves priority (Adshead, Mackie and Paetkau, 1963). However, the animals have been cultured for several years as *H. pseudoligactis* and this name will be continued to be used until it can be shown that the species is the same as that Rowan described as *H. canadensis* (Rowan, 1930). These species were chosen since they represent the extremes on a continuum of behavior and electrical activity of ten species of hydra thus far investigated. The contraction pulse activity of *H. pirardi* consists predominately of bursts of potentials while that of *H. pseudoligactis* consists almost exclusively of single events. Under conditions of external stimulation, however, the electrical activity of the latter species may be changed to give a temporal pattern of bursts of potentials similar to that of *H. pirardi.*

This investigation is a first step in an effort to construct testable models for the production of potentials by pacemaker systems in hydroids. The current study was undertaken to quantify the characteristics of inter-pulse intervals in two selected species of *Hydra.* The characteristics of the interval measurements will be compared in subsequent studies with those observed for other hydroids, and with those of intervals generated using theoretical models.

Materials and Methods

The conditions employed for culturing the hydra were similar to those described by Loomis and Lenhoff (1956). Animals were raised in a medium consisting of glass distilled water to which 1.5×10^{-5} M $CaCl_2$, 1.2×10^{-3} M $NaHCO_3$, and 1.2×10^{-4} M Na_4 EDTA had been added. The hydra were fed daily with a plentiful supply of freshly hatched *Artemia salina* nauplii. The temperature was held at 21 ± 1° C and the animals were reared in constant illumination, approximately 30 foot candles.

The electrical recordings were from hydra randomly selected from the culture dishes 24 hours after feeding. Continuous recordings of the spontaneous electrical activity of individual hydra were made using a glass suction electrode (Josephson, 1967). The individual recording periods were 10 to 12 hours in duration. The electrode has a bell-shaped opening 0.4–0.6 mm in internal diameter about 1 mm from the open end. The other end of the electrode is attached to a syringe by means of flexible tygon tubing and is held in a micromanipulator. The hydra is lightly sucked into the open end of the electrode, and sits with its base in the bell-shaped opening (Fig. 1). The electrode is filled with culture solution and contains a silver wire inserted through the tubing to increase the conductivity. Electrical contact in this system is made between the tissue of the hydra base, the culture medium and the silver wire. A reference electrode consists of a coil of silver wire approximately 5 cm from the recording electrode. Electrical potentials from the preparation are amplified using capacitor-coupled amplifiers having long time constants, with the final display on an oscilloscope and penwriter. Both electrodes are placed in 200 ml of culture solution in a finger bowl. The recording electrode is positioned so that the hydra touches neither the bottom of the dish nor the upper surface film with its tentacles.

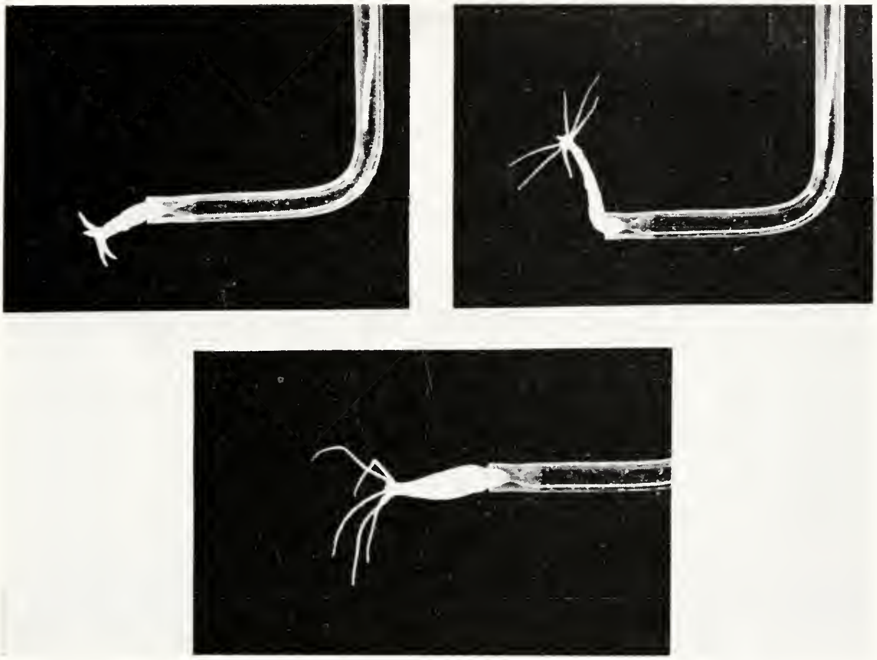

FIGURE 1. Hydra placed in the recording electrode: upper left: *H. pirardi* in a contracted state; upper right: *H. pirardi* in an elongated position; lower: *H. pseudoligactis* in a normal relaxed position.

It was found that increased suction on the base of the hydra in the electrode affected both the frequency and temporal patterning of pacemaker activity. Therefore, during the recording periods the suction was kept as low as seemed feasible. Occasionally because of the light suction on the electrode, the hydra would pull its base out of the electrode during the recording period. It was found possible to feed hydra attached to the electrode and have them produce buds over several days while still recording their pacemaker activities.

Visual observations using a dissecting microscope were made of the overt behavior of the hydra while simultaneously monitoring its electrical activity. Column contractions were manually recorded during phases of the electrical recordings using a telegraph key which activated a channel of the penwriter. Such visual observations were made at the start and termination of a 12 hour record of electrical activity to ensure correspondence between the recorded pulses designated as CP's and column contractions of the animal.

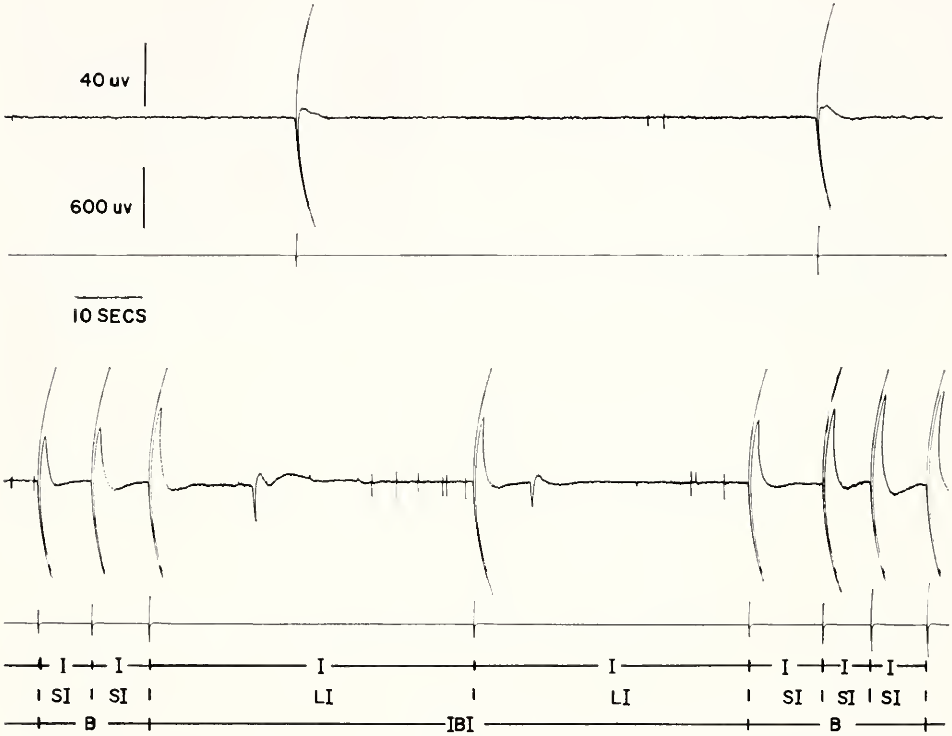

FIGURE 2. Electrical records of hydra. Upper two records: *H. pseudoligactis*, lower two records: *H. Pirardi*, for definition of the symbols see the text. The positive polarity is in the upward direction in this and other figures of electrical recordings.

In order to distinguish between the behavioral event of column contraction and the associated electrical event the following terms are used: (1) a column contraction designates a single contraction of the body column which may be visually observed, or recorded using a transducer as described by Josephson (1967); (2) a contraction burst is a behavioral event consisting of a series of individual column contractions, each contraction in the series separated by less than 20 seconds from a subsequent or preceding column contraction in the burst. At the termination of a contraction burst the hydra appears as a tight ball with its column fully contracted; and (3) a Column Contraction Pulse (designated here as a Contraction Pulse, or CP) is a single electrical potential preceding a column contraction by 0.1 to 0.2 seconds. The electrical correlation of a contraction burst is a burst of Contraction Pulses (a CP burst).

Measurements were made from the penwriter records of the intercontraction pulse interval lengths to an accuracy of 0.4 seconds. Both inter-pulse interval histograms and joint interval scatter diagrams were used to characterize the distributions of the inter-pulse intervals. These methods are described later in the results section.

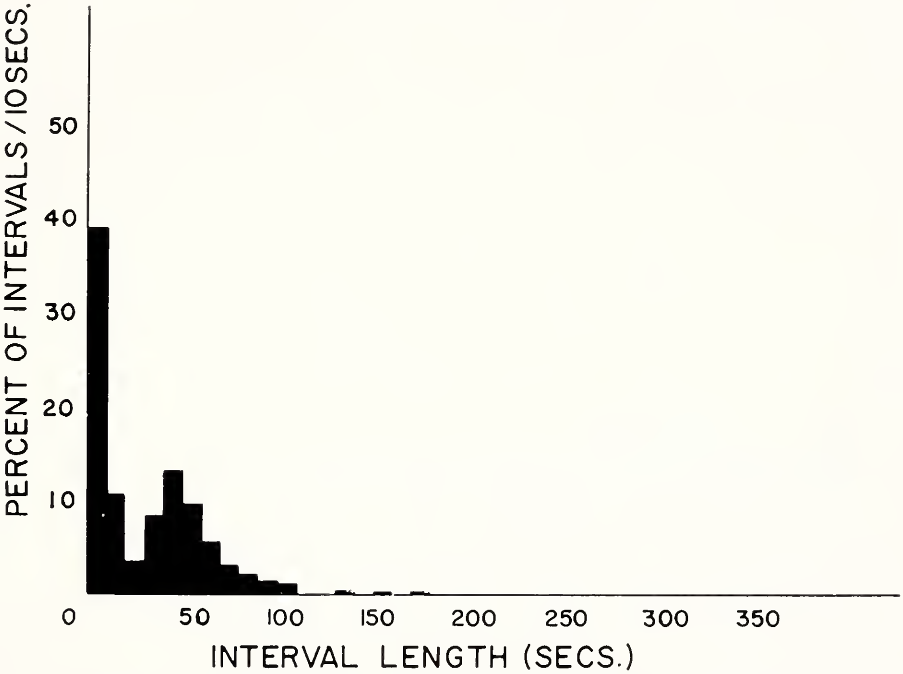

FIGURE 3. Inter-pulse interval histogram for *H. pirardi*. The intervals are from a 10 hour record from a single animal.

With *H. pseudoligactis*, the CP system was sometimes induced to fire predominately in bursts rather than as single CP's by: (1) increasing the electrode suction; (2) giving intermittant light stimulation; or (3) exposing the animal to live *Artemia salina* nauplii. The CP pattern was determined for three different levels of suction: 0, 10, 20 cm of water, as measured by a manometer attached to the tygon tubing of the recording electrode. Intermittant light stimulation consisted of successive exposures of the hydra to two minutes of "strong light" and two minute periods of ambient light. The "strong light" was supplied by an American Optical Lamp, Model 353 which housed a G. E. bulb, model 1493, operated at 6 volts. The bulb was placed 15 cm from the preparation and light was directed through a heat filter and the culture solution onto the whole hydra. The CP activity was monitored during 1½ hour period of intermittant light stimulation and compared with that for a similar period following the termination of such stimulation. Contraction Pulses were recorded from unstimulated hydra and their temporal patterns compared with those attained after the animal had fed to satiation on artemia. The nauplii were introduced one at a time into the medium above the hydra, creating minimal mechanical disturbance to the preparation.

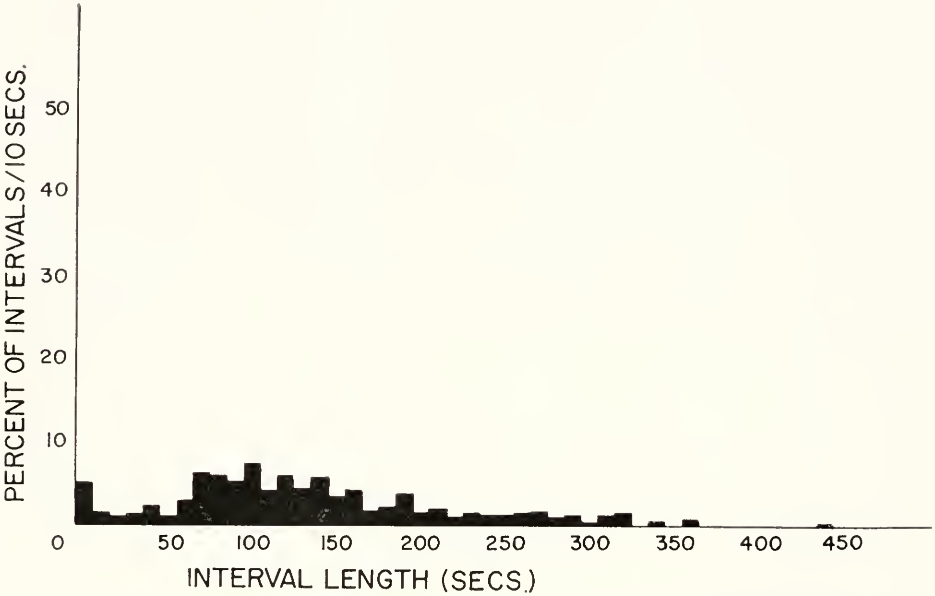

FIGURE 4. Inter-pulse interval histogram for *H. pseudoligactis.* The intervals are from a 10 hour record from a single animal.

RESULTS

Temporal patterns of spontaneous Contraction Pulses

Inter-species differences. The temporal patterns of spontaneous Contraction Pulses were markedly different in the two species. Figure 2 shows a 140 second portion of a continuous record from a single *H. pseudoligactis* (upper pair of records), and a *H. pirardi* (lower pair of records). The upper channel of both pairs of records has greater sensitivity and was used to record both the CP's which are the large potentials, as well as Rhythmic Potentials (RP's) and Tentacle Contraction Pulses (TCP's) which are much smaller potentials. These smaller pulses are clearly evident in the record of *H. pirardi.* At the sensitivity used to record RP's and TCP's considerable truncation of the CP amplitude occurs in the penwriter records. Thus, the second channel was used at a lower sensitivity to record undistorted CP's unaccompanied by the smaller potentials.

H. pseudoligactis gave CP's predominantly as single widely spaced events. While *H. pirardi* gave both single pulses and bursts of potentials. When the interval lengths between successive CP's for all intervals in a 10 hour record were plotted in the form of a percentage histogram, the resulting distribution was clearly bimodal in the case of *H. pirardi* (Fig. 3). The lower modal class, consisting of approximately 40 per cent of the intervals for this preparation, represents short intervals occurring within bursts, while the upper modal class (about 13 per cent of the intervals) represents longer intervals not in the bursts. The trough between the two modes was used as a criterion to classify intervals into long and short intervals in the electrical recordings. On the basis of such histograms it was found that 20 seconds was a suitable cut-off value to characterize intervals as belonging to the long or short class. A burst was defined as consisting of two or more successive pulses separated by short intervals (SI). An inter-burst interval (IBI) was defined as the sum of all long inter-

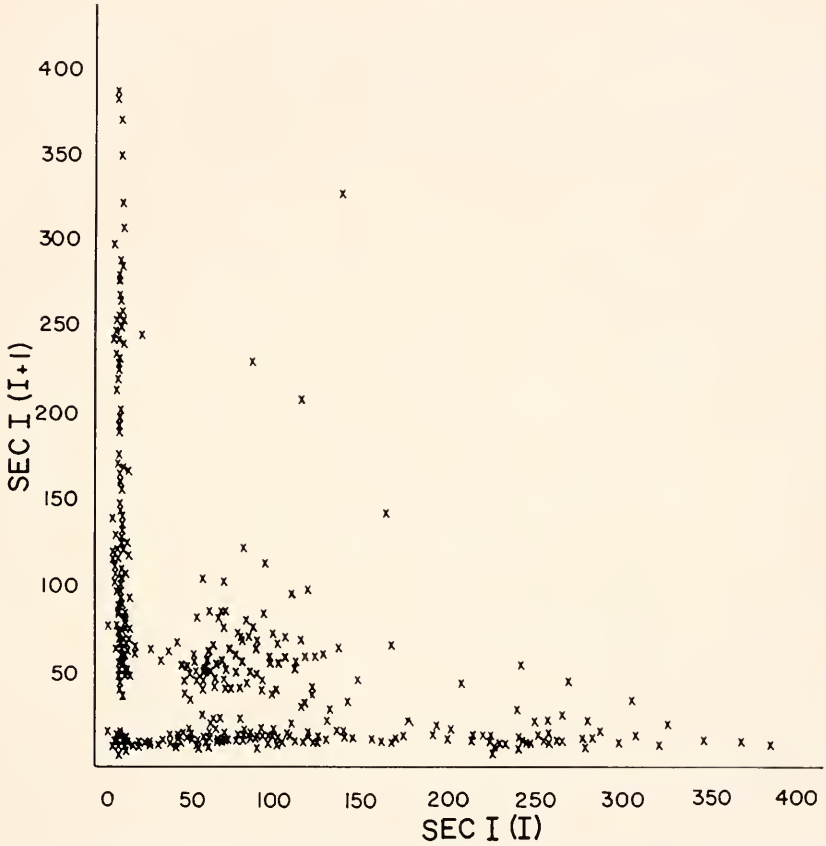

FIGURE 5. First order joint interval scatter diagram for *H. pirardi*. For further details see text. The preparation is the same as that for Figure 3.

vals (LI) between two adjacent bursts. The segment of the *H. pirardi* record depicted in Figure 2 consists of seven inter-pulse intervals, five of which are short intervals while the remaining two are long intervals. The record contains two bursts and one inter-burst interval.

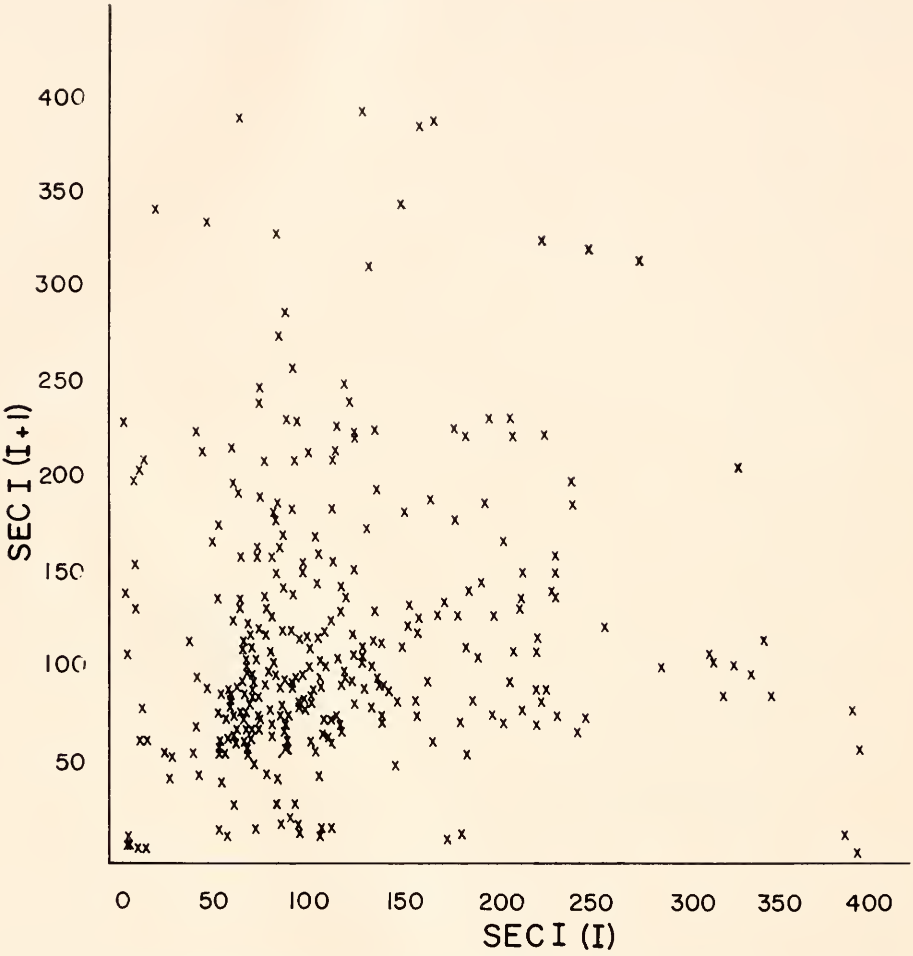

Figure 6. First order joint interval scatter diagram for *H. pseudoligactis*. For further details see text. The preparation is the same as that for Figure 4.

In contrast to the histograms for *H. pirardi* which contained a large percentage of short intervals (about 50 per cent for the preparation shown in Figure 3), those of *H. pseudoligactis* did not show bimodality and had few short intervals. The inter-pulse histogram for all intervals in a 10 hour recording period for the *H. pseudoligactis* preparation previously described, is plotted in Figure 4. In this case only 9 per cent of the intervals are short intervals (*i.e.*, less than 20 secs) indicating that there are few bursts for this animal.

The temporal patterns of CP's for the two species were compared using a second graphical procedure, a joint interval scatter diagram. This method has been used to quantitatively describe the spontaneous activity of a single neuron (Rodiech, Kiang and Gerstein, 1962). In Figure 5 the first order joint interval scatter diagram is plotted for the *H. pirardi* preparation. This is a graph of each successive inter-CP interval plotted as the ordinate measure against the inter-CP interval of the preceding interval as the abscissa. Thus the $i + 1$th interval is plotted against the ith interval, giving a graph of $n - 1$ points from a record of n inter-CP intervals. As is seen for this preparation, records containing a large number of bursts give characteristic L-shaped plots. Points close to the origin constitute intervals between pulses within a burst. Those near the abscissa and parallel to it are intervals which precede bursts; those near to the ordinate depict intervals following a burst. In contrast to this graph, the first order joint interval scatter diagrams for the *H. pseudoligactis* shows points widely dispersed and roughly symmetrically distributed about 45° line through the origin (Fig. 6). The graphs for all ten preparations of *H. pseudoligactis* were quite similar to that shown here, and were quite different from the L-shaped plots characterizing the burst patterns of the ten specimens of *H. pirardi*.

TABLE I

Measurements of intervals between Contraction Pulses for H. pirardi *and* H. pseudoligactis. *The data are from 10–12 hour recordings for 10 hydra of each species*

Measurement	Mean (Standard error)		
	H. pirardi	*H. pseudoligactis*	
Number of intervals/12 hr	1167 (137)	397 (30)	$P < 0.001$
Short interval (sec)	9.0 (0.9)	6.3 (0.1)	$P < 0.05$
Long interval (sec)	94 (14)	128 (8)	$P < 0.05$
Per cent of intervals classified as long intervals	42 (4)	89 (3)	$P < 0.001$
Number of bursts/12 hr	279 (29)	26 (6)	$P < 0.001$
Number of intervals/burst	2.5 (0.3)	1.7 (0.1)	$P < 0.05$
Mean interval/burst (sec)	9.3 (0.9)	7.0 (1.0)	$P > 0.10$
Burst lengths (sec)	20.7 (1.4)	10.8 (1.1)	$P < 0.001$
Inter-burst interval (sec)	151 (20)	1746 (336)	$P < 0.001$

The temporal patterns of the contraction potential records from the two species were further compared with measurements utilizing long and short intervals and the derived quantities of bursts and inter-bursts. A summary of these measurements from the ten hydra of the two species is given in Table I. Mean values together with their standard errors are presented for nine parameters of Contraction Pulse activity. It is evident from this table that the overall frequency of CP's, as measured by the number of intervals/12 hours is much greater for *H. pirardi*. This is a result of a greater number of bursts and therefore a smaller inter-burst interval length than for *H. pseudoligactis*.

When the burst characteristics of the two species are compared it is noted that the burst lengths of *H. pirardi* are longer. This results from both a larger number of intervals per burst and a greater mean interval per burst for this species. Differences in the distributions for burst and inter-burst characteristics were investigated in order to determine statistical measures which most clearly distinguished between the two species. It is seen from Figure 7 that two measurements, the number of bursts/12 hours and the burst length may be used to separate animals of the two species into non-overlapping sets. Thus, we see that for these two species the temporal characteristics of the column contraction pacemaker potentials might be used as criteria for taxonomic classification.

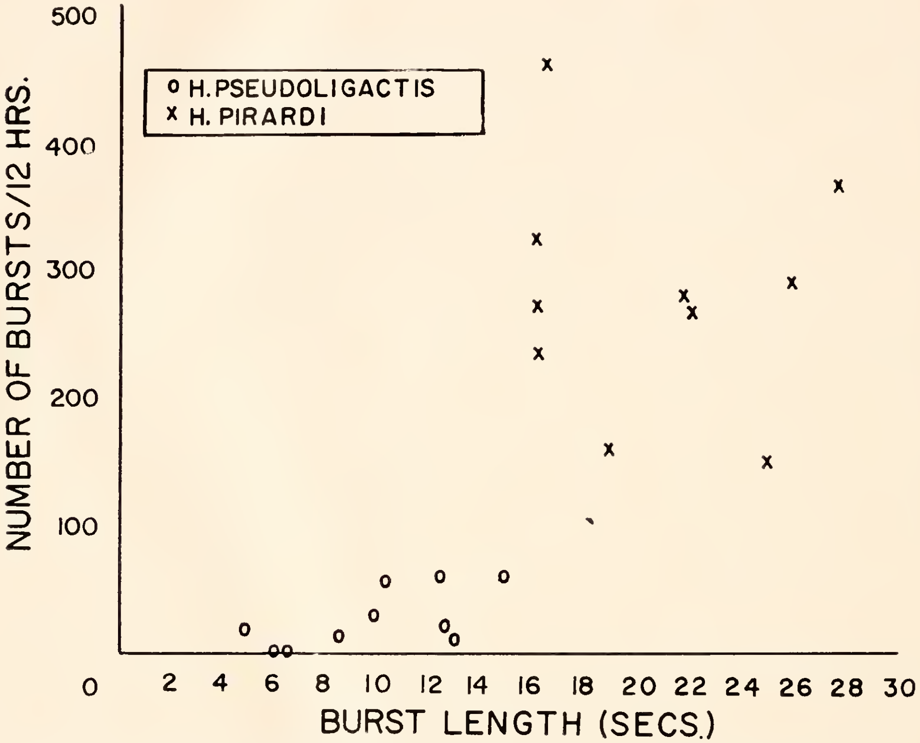

FIGURE 7. Graph of the number of brusts/12 hours and the burst length for ten preparations of *H. pseudoligactis* (open circles) and *H. pirardi* (crosses).

Characteristics of bursts of contraction pulses: (a) Amplitudes of CP's within bursts. Measurements were made of some additional characteristics of the bursts of *H. pirardi.* There were insufficient bursts in the records of *H. pseudoligactis* to apply statistical analyses. However, the general relationships observed for *H. pirardi,* appear to apply to the records of spontaneously produced CP bursts in *H. pseudoligactis,* and are quite evident when animals of this species are stimulated to give bursts of potentials.

An easily discernible feature of bursts of potentials is the increase in the amplitudes of successive CP's in a burst. Both Passano and McCullough (1964) and

Josephson (1967) noted that CP's are compound potentials consisting of both positive and negative phases when recorded externally. In the present study the pulses contain both positive and negative phases (and also an after potential due to capacitor discharge in the capacitor-coupled recording system). The initial positive and negative components were measured in the long-term records. In preparations of *H. pirardi* the amplitudes of both the negative and positive components of the potentials were found to increase for successive pulses in a burst. Except for one of the ten preparations studied, in which the amplitudes of the first two pulses in a burst were approximately the same, a successive increase in the amplitude of CP's in a burst was a constant feature of the records. In Table II, the relationship of the CP amplitudes and the position of the pulse in a group of CP's is given for the *H. pirardi* preparation previously described. Analysis of variance tests indicated that the increase in both phases of CP's within a burst is statistically significant ($P < 0.05$).

TABLE II

Amplitude of a CP and position of the CP in a group of CP's for H. pirardi. *The data are from a 12 hour recording from a single animal*

Number of pulses per group	Mean amplitude (mv): Positive phase / Negative phase — Position of pulse in the group: 1	2	3	4	Number of groups
1	1.93 1.44				96
2	1.77 1.46	2.49 1.80			87
3	1.81 1.42	2.53 1.76	3.18 2.44		49
4	1.88 0.82	2.53 2.06	3.17 2.90	3.64 3.53	27

Characteristics of bursts of contraction pulses: (b) Lengths of short intervals within a burst and long intervals within an inter-burst. Passano and McCullough (1964) observed that spontaneous CP bursts in *H. pirardi* lasted less than 30 seconds and consisted of from 5 to 8 pulses. They noted that the inter-CP interval lengths in a burst were shorter at the mid-point of the burst than either at the start or end of the burst.

Analyses were made of the inter-pulse intervals in recordings of *H. pirardi*, to determine both the interval lengths of short intervals within a burst, and the lengths of long intervals within an inter-burst interval. Data were pooled for 10 animals and the mean lengths were determined for intervals at various positions in a burst and in an inter-burst. The relationship between the interval length and the position of the interval in a burst is given for bursts of various sizes

TABLE III

Interval length and position of interval in a burst for H. pirardi. *The data are based on 10–12 hour recordings of each of 10 individual animals*

Number of short intervals per burst	Mean interval (sec)					Number of bursts
	Position of interval in the burst					
	1	2	3	4	5	
1	10.4					860
2	10.1	9.8				789
3	9.5	7.4	8.5			597
4	8.5	6.0	5.7	6.9		392
5	8.7	5.7	5.2	5.5	6.9	302

in Table III. Although in our recordings of *H. pirardi* the burst lengths were smaller (about 20 seconds, see Table I) and consisted of fewer pulses (2 to 5 usually), the characteristic pattern of interval lengths within a burst observed by Passano and McCullough (1964) was evident. Interval lengths were shortest in mid-burst than in the early stages or at the termination of a burst. The length of the first short interval was significantly greater ($P < 0.001$) than the lengths of other intervals in the burst.

Table IV reveals the relationship between the length of a long interval and its position in the inter-burst interval. The first long interval is significantly longer ($P < 0.001$) than the subsequent long intervals in the inter-burst interval. However, there is a systematic decrease in the lengths of progressive intervals in the inter-burst interval. Thus, the temporal pattern of short intervals within a burst is quite different from that of long intervals within an inter-burst interval.

Modifications of CP patterns in H. pseudoligactis *induced by external stimulation*

The previous results indicate that in *H. pirardi,* there are normally many spontaneous bursts of CP's contrasted with *H. pseudoligactis* in which bursts of CP's are rare events in unstimulated preparations. Any models devised for pacemaker

TABLE IV

Interval length and position of interval in an inter-burst interval for H. pirardi. *The data are from 10–12 hour recordings for each of 10 individual animals*

Number of long intervals per inter-burst interval	IBI (sec)	Position of interval in the inter-burst interval					Number of inter-burst intervals
		1	2	3	4	5	
1	106	106					1595
2	145	93	52				983
3	205	86	66	53			246
4	244	78	59	62	45		77
5	356	88	74	70	66	58	27

activity in *Hydra* should include provisions for both types of pulse patterns. *H. pseudoligactis* will produce bursts when appropriately stimulated. Effective stimuli include: (1) increased suction of the hydra in the recording electrode; (2) intermittant light stimulation of the whole preparation; and (3) exposure to stimuli inducing a feeding reaction in the animal. When *H. pseudoligactis* is induced to give bursts of CP's, their temporal patterns are similar to those previously noted for preparations of *H. pirardi*.

(1) *The effect of increased electrode suction.* In the course of long-term recordings of the spontaneous activity of several species of *Hydra,* it was observed that animals damaged during placement in the electrode produced many RP's. McCullough (1965) also noted an increased RP frequency in hydra placed in stressful recording situations. For animals such as *H. pirardi,* which normally produce many bursts of CP's, the initial effect is an elongation of the inter-CP burst interval accompanied by more pulses per burst and longer burst lengths. On the other hand, for hydra usually giving single CP's, such as *H. pseudoligactis,* the effect is to change the CP pattern to one primarly of burst of potentials.

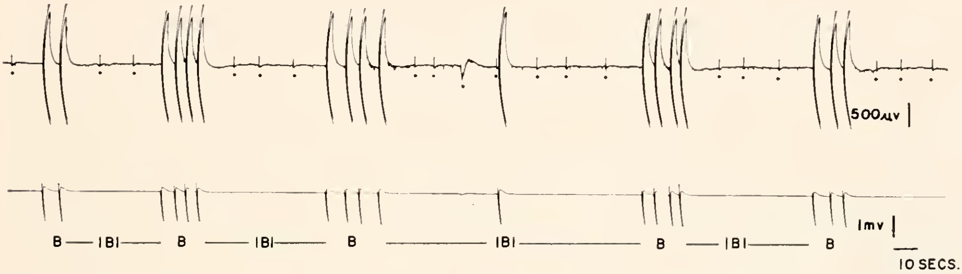

FIGURE 8. Electrical record of *H. pseudoligactis* under high electrode suction (20 cm of water).

In order to study the induction of bursts in *H. pseudoligactis* more systematically preparations were exposed to three different levels of suction in the recording electrode (0, 10, 20 cm of water). It was found that either intermediate (10 cm) or high (20 cm) pressure induced bursts of CP's accompanied by enhanced RP frequencies during inter-CP burst intervals. An example of an electrical record of a preparation at the higher pressure is given in Figure 8. Both the CP bursts and single RP's are easily discernible in the upper channel recording, and contrast markedly with the records characteristic of unstimulated *H. pseudoligactis* (see upper records of Figure 2).

In Table V, nine measurements of inter-CP intervals are compared for the three pressure regimes. As the suction on the base of the hydra is increased there is a significant increase in the frequency of CP bursts ($P < 0.01$), and therefore a marked reduction in the inter-burst interval length. There is significant decrease in the per cent of intervals which are long intervals, and also a reduction in the long interval length ($P < 0.01$). However, while increased pressure results in more short intervals within a burst ($P < 0.05$), the mean interval per burst is not affected significantly ($P > 0.10$).

CP bursts in *H. pseudoligactis* induced by increased electrode suction have the same characteristics as those of unstimulated *H. pirardi:* (1) both positive and negative phases of the CP amplitudes increase progressively with successive pulses in a burst; (2) intervals are shorter in mid-burst than at the start or end of the burst; and (3) long intervals significantly decrease in length with successive positions in the inter-burst interval.

(2) *The effect of intermittant light stimulation.* Several studies have shown that the effect of light on *Hydra* is complex, giving rise to both inhibition and excitation of pacemaker systems (Passano and McCullough, 1962, 1964, 1965; Rushforth, 1967a). When *H. pirardi* is stimulated with light for two minutes (about 600 ft-c), followed by two minute periods of ambient light (about 30 ft-c), the CP pattern is altered. The normal burst pattern is changed to one in which bursts occur exclusively during the two minute periods of direct illumination (Rushforth, 1967a). In contrast when preparations of *H. pseudoligactis* are exposed to this regime, CP's are inhibited during light stimulation. On direct illumination of this species, the frequencies of both RP's and TCP's are increased. During the interposed periods of ambient light, however, the CP System is induced to fire in bursts. For a sample of 80 periods of illumination, the CP frequency was significantly smaller (0.15 ± 0.04 versus 5.45 ± 0.17, $P < .01$) and the RP frequency significantly greater (7.15 ± 0.19 *versus* 1.34 ± 0.14, $P < 0.01$) compared with control periods of ambient light.

TABLE V

Effects of pressure on CP interval measurements in H. pseudoligactis. *The data are based on a one hour recording at each pressure for each of eight animals*

Measurement	Mean (Standard error)		
	Low pressure (0 cm)	Intermediate pressure (10 cm)	High pressure (20 cm)
Number of intervals/12 hr	260 (37)	438 (59)	930 (376)
Short interval lengths (sec)	9.4 (1.2)	9.7 (5.8)	11.0 (0.7)
Long interval lengths (sec)	201 (28)	160 (27)	130 (29)
Per cent of intervals classified as long intervals	94 (8)	61 (7)	36 (10)
Number of bursts/12 hr	10 (4)	62 (7)	165 (82)
Number of intervals/burst	1.2 (0.2)	2.0 (0.5)	1.8 (0.4)
Mean interval length/burst (sec)	10.4 (1.3)	10.8 (0.8)	11.3 (0.7)
Burst lengths (sec)	11.7 (2.4)	18.1 (3.0)	20.5 (5.3)
Inter-burst interval lengths (sec)	4653 (1095)	655 (97)	272 (99)

An analysis of the stimulated bursts in the ambient light period showed the short interval lengths depended on their positions in the burst in a similar manner to spontaneously produced bursts in *H. pirardi*, or bursts in *H. pseudoligactis* stimulated by increased electrode suction. The intervals are shorter in mid-burst.

(3) *Effects of feeding.* In previous studies of the contraction behavior of various species of *Hydra,* it was observed that feeding stimuli inhibited spontaneous contractions and those induced by external stimuli (Rushforth, 1967a). Subsequent experiments in which both the contraction behavior and correlated electrical activity were simultaneously monitored showed that following CP inhibition during

feeding of an *Artemia* or exposure to the tripeptide reduced glutathione the CP frequency was increased significantly before returning to prestimulus levels. During a period of approximately 1 hour of increased CP activity, the temporal pattern of electrical pulses of *H. pseudoligactis* were quantified by comparing interpulse measurements taken before and after artemia ingestion. After feeding there are relatively fewer long intervals and considerably more bursts than before exposure to *Artemia*, for comparable time periods. There is a significant reduction both in the long and short interval lengths ($P < 0.01$), while the number of pulses per burst is significantly increased after ingestion of the artemia ($P < 0.01$).

The bursts induced by feeding have the same general features as those elicited by light or by increased electrode suction, or as those spontaneously produced by *H. pirardi*. Short intervals are shortest in mid-burst, long intervals decrease systematically within an inter-burst, and the amplitudes of pulses increase in size either throughout the burst or in the initial part of the burst levelling off to a constant value in the late stages.

Discussion

Passano and McCullough (1964) described several characteristics of the pacemaker system of coordinating periodic contractions of the body column in *Hydra* in some detail. In observations of three species (*H. littoralis*, *H. pirardi*, and *H. carnea*) they emphasized that electrical output of this system consisted primarily of bursts of potentials. However, they noted that single contractions, causing only a slight shortening of the animal, comprise approximately 10 per cent of the hydra's contraction behavior. In the current study the temporal pattern of burst of potentials for undisturbed species of *H. pirardi* has been contrasted with the pattern found in *H. pseudoligactis*. In this latter species unstimulated hydra give primarily single, widely spaced pulses and only occasionally produce bursts of potentials. However, when these animals are stimulated, the temporal pattern is changed to one largely consisting of bursts.

Three features of the interval patterns were characteristic for unstimulated *H. pirardi* and for *H. pseudoligactis*, induced to produce bursts: (1) amplitudes of pulses increased with successive positions in a burst; (2) intervals were shorter in mid-burst than at the start or termination of the burst; and (3) intervals decreased in length with successive positions in the inter-burst interval. These three features have been observed in other species of *Hydra* (Passano and McCullough, 1964, Rushforth, unpublished) and appear inherent properties of the temporal CP pattern in this coelenterate.

In studies of the electrical properties of the body wall of *H. oligactis*, Josephson and Macklin (1967, 1969) indicate that CP's are generated by the epithelial cells. They suggest that the inner membranes of the ectodermal epitheliomuscular cells are sites of CP production. Strumwasser (1967) suggested that the process generating periodic bursts of spikes in a neuron could be explained on the basis of an internal oscillator. "The frequency of his internal oscillator determines the burst frequency, whereas the interaction between the amplitude of the oscillator and a fixed discharge threshold determines the length of the burst and the number of spikes in it" (Strumwasser, 1967, page 306). Thus long intervals (between bursts) and short intervals (within bursts) in *Aplysia* are thought to result from a single generating mechanism.

In *Hydra*, the interval lengths of long intervals were found to have quite a different functional relationship to their position in an inter-burst interval from that of short intervals within bursts. Both in unstimulated preparations of *H. pirardi*, and in *H. pseudoligactis* induced to give bursts of potentials by increased electrode suction the intervals decrease successively in length throughout the inter-burst interval. The interval immediately following a burst was significantly longer than the subsequent intervals prior to the next burst. The different patterns suggest that different mechanisms seem to be involved in the production of short and long intervals.

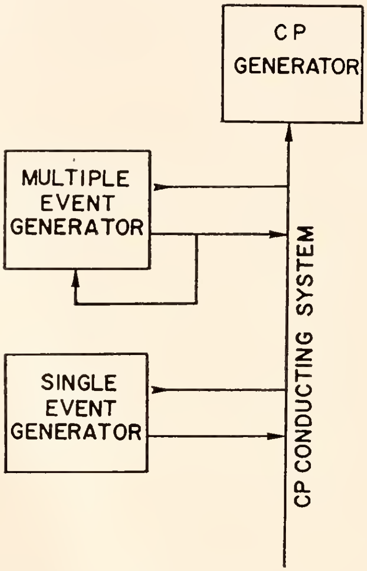

FIGURE 9. Schematic model of the CP system of *Hydra*. An arrow indicates excitatory input, a reverse arrow indicates inhibitory input.

The following model is offered as one which could account for the observed CP patterns. The proposed model is shown schematically in Figure 9. There are two elements, one producing single events and another producing multiple events. Both elements trigger the CP conducting system and thus initiate CP's. The multiple event generator has short term excitation to itself, such excitation leads to firing in bursts, and is also responsible for the decrease of interval lengths in the initial phases of the burst. There is also inhibitory feedback to the multiple event generator, probably mediated *via* the CP conducting system. This inhibitory feedback is slow rising, and the inhibitory effects of several CP's in succession are accumulative. The growing inhibition within a burst leads to increased intervals toward the end of the burst and eventually the termination of the burst. The joint effect of the excitatory and inhibitory components gives rise to the observed pattern of intervals within a burst, *i.e.* intervals are shorter in mid-burst than at the start or end of the burst.

The inhibitory effect of single CP's upon the multiple event generator can be directly demonstrated by driving the CP System with single stimuli. Exciting the CP System once each 15 to 30 seconds with single electric shocks or pulses of mechanical stimuli inhibit CP bursts (Passano and McCullough, 1964, Rushforth and Burke, in preparation). Indirect evidence for the inhibition of CP bursts by CP singles may be derived from an analysis of inter-CP intervals in long-term records consisting of both single pulses and bursts. The inter-burst interval is significantly longer ($P < 0.01$) if it includes a CP single than if no CP is present in the interval. With increasing numbers of single CP's the inter-CP burst interval is correspondingly increased in length (see Table IV).

It is proposed that there is inhibitory feedback to the single event generator mediated by the CP conducting system. Such inhibition results in the decrease in the intervals between single CP's following a burst of CP's throughout the inter-burst interval. This suggests gradual escape of the single event generator from long lasting inhibition following a CP burst.

The model proposed for the CP System is incomplete. Modifications of the model are necessary to take into account interactions of the CP System with both the Rhythmic Pulse System and the Tentacle Pulse System. The model is likely to be further modified to take into account the effects of external stimuli on the pacemaker system. Photic, mechanical and chemical stimuli are likely to have complex influences on the pacemaker systems, possibly affecting more than one component.

A major problem, as with all other hydroids, is the identification of the cellular elements involved in the pacemaker and conduction activities. Passano and McCullough (1965) proposed that the pacemaker system for CP's was the ectodermal nerve net. The size of the CP's, however, seemed too large to be exclusively the result of activity from the small dispersed neurons in the nerve net. Josephson and Macklin (1967, 1969) demonstrated that *Hydra* has a transepithelial potential. The fluid in the body cavity is typically 15 to 40 mv positive with respect to the outer bathing solution. Superimposed on this transepithelial potential are the negative going (depolarizing) CP's. It was hoped initially that direct measurement of the transepithelial potential and possible triggering of CP's in response to depolarizing current pulses might provide direct measurements for the construction of models for the production of CP's. However, CP's were found to have several surprising features in relation to the transepithelial potential which made such efforts unfeasible. Josephson and Macklin (1969) found: (1) the amplitude and frequency of CP's are essentially independent of the transepithelial potential when the latter is altered by imposed current; (2) there is little change in the impedance of the hydra column during CP firing; (3) when the transepithelial potential is clamped at zero, CP's continue to appear spontaneously as current spikes. There are four membranes between the inside of the body cavity and the outside solution, only one of which is likely to be the CP-generating membrane. Josephson and Macklin have shown that the CP-generating membrane forms only a small fraction of the total transverse impedance of the column. This unfortunately means that the CP-generating membrane cannot adequately be studied by measuring potentials across the body wall, or passing current through it, since the presence of large series impedance elements masks the properties of the CP-generating membrane.

The mechanisms by which CP's are conducted along the hydra column are unknown. Activity in the nerve net may trigger epithelial cells to produce these large electrical potentials. However, nerve free epithelia capable of producing propagated electrical pulses have been found in hydrozoans (Mackie, 1965, Mackie and Passano, 1968), so that it is possible that CP conduction as well as initiation is due to properties of the epithelial cells. In the present study the same characteristics of contraction potentials have been investigated in two species of *Hydra*. These characteristics impose boundary conditions on the parameters for models of pulse production. In subsequent papers we will investigate the properties of isolated hydroid pacemaker systems and the interactions of such pacemakers in the intact animal.

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SUMMARY

1. The temporal patterns of spontaneously produced Contraction Pulses (CP's) were investigated in two species of *Hydra*. It was found that *H. pseudoligactis* frequently give CP's as single, widely spaced events, whereas, *H. pirardi* produce many CP bursts. Inter-pulse interval histograms and joint order scatter diagrams, constructed on the basis of 10 to 12 hour recordings from 10 preparations of the two species, were employed to depict these contrasting temporal patterns of pulse production. The burst frequency and the burst length for CP's were found to be so different for the two species that these measurements alone allowed a unique designation of the animals into the two species classes.

2. CP bursts from unstimulated *H. pirardi* had the following characteristic features: (a) both positive and negative phases of the CP amplitudes increased progressively with successive pulses in a burst; (b) intervals were shorter in mid-burst than at the start or end of the burst; and (c) intervals progressively decrease in length with successive position in an inter-burst interval.

3. The CP pattern of *H. pseudoligactis* can be changed from one primarily of single CP's to one consisting predominantly of bursts of pulses by the following external stimulation: (a) increased electrode suction; (b) intermittant light stimulation; and (c) exposure to feeding stimuli. The three features of CP bursts found in unstimulated *H. pirardi*, are exhibited by the CP burst pattern induced by such stimuli in *H. pseudoligactis*.

LITERATURE CITED

ADSHEAD, P. C., G. O. MACKIE AND P. PAETKAU, 1963. On the *Hydras* of Alberta and the Northwest Territories. *Bull. Nat. Mus. Canada*, **199**: 1–13.

DAVIS, W. J., AND R. K. MURPHY, 1969. Discharge patterns of swimmeret motoneurones in the lobster, simulated with a digital computer. *J. Exp. Biol.*, **50**: 119–128.

FULTON, C., 1963. Rhythmic movements in *Cordylophora*. *J. Cell. Comp. Physiol.*, **61**: 39–52.

IKEDA, K., AND C. A. G. WIERSMA, 1964. Autogenic rhythmicity in the abdominal ganglia of the crayfish: the control of swimmeret movements. *Comp. Biochem. Physiol.*, **12**: 107–115.

JOSEPHSON, R. K., 1967. Conduction and contraction in the column of hydra. *J. Exp. Biol.*, **47**: 179–190.

JOSEPHSON, R. K., AND G. O. MACKIE, 1965. Multiple pacemakers and the behavior of the hydroid *Tubularia*. *J. Exp. Biol.*, **43**: 293–332.

JOSEPHSON, R. K., AND M. MACKLIN, 1967. Transepithelial potentials in *Hydra*. *Science*, **156**: 1629–1631.

JOSEPHSON, R. K., AND M. MACKLIN, 1969. Electrical properties of the body wall of *Hydra*. *J. Gen. Physiol.*, **53**: 638–665.

LOOMIS, W. F., AND H. M. LENHOFF, 1956. Growth and sexual differentiation of hydra in mass culture. *J. Exp. Zool.*, **132**: 555–574.

MACKIE, G. O., 1965. Conduction in the nerve-free epithelia of siphonophores. *Amer. Zool.*, **5**: 439–453.

MACKIE, G. O., 1968. Electrical activity in the hydroid *Cordylophora*. *J. Exp. Biol.*, **49**: 387–400.

MACKIE, G. O., AND L. M. PASSANO, 1968. Epithelial conduction in hydramedusae. *J. Gen. Physiol.*, **52**: 600–621.

MCCULLOUGH, C. B., 1965. Pacemaker interaction in *Hydra*. *Amer. Zool.*, **5**: 499–504.

MORIN, J. G., 1969. Mechanisms controlling behavior and bioluminescence in the colonial hydroid *Obelia*. *Ph.D. thesis, Harvard University*, Cambridge, Massachusetts, 156 pp.

PASSANO, L. M., AND C. B. MCCULLOUGH, 1962. The light response and the rhythmic potentials of hydra. *Proc. Nat. Acad. Sci. U. S. A.*, **48**: 1376–1382.

PASSANO, L. M., AND C. B. MCCULLOUGH, 1963. Pacemaker hierarchies controlling the behavior of hydras. *Nature*, **199**: 1174–1175.

PASSANO, L. M., AND C. B. MCCULLOUGH, 1964. Coordinating systems of behavior in *Hydra*. I. Pacemaker systems of the periodic contractions. *J. Exp. Biol.*, **41**: 643–664.

PASSANO, L. M., AND C. B. MCCULLOUGH, 1965. Coordinating systems and behavior in *Hydra*. II. The rhythmic potential system. *J. Exp. Biol.*, **42**: 205–231.

RODIECK, R. W., N. Y. S. KIANG AND G. L. GERSTEIN, 1962. Some quantitative methods for the study of spontaneous activity of single neurons. *Biophys.*, **2**: 351–368.

ROWAN, W., 1930. On a new hydra from Alberta. *Trans. Roy. Soc. Canada*, **24**: 165.

RUSHFORTH, N. B., 1966. An analysis of spontaneous contraction pulse patterns in *Hydra*. *Amer. Zool.*, **6**: 524.

RUSHFORTH, N. B., 1967a. Chemical and physical factors affecting behavior in *Hydra:* interactions among factors affecting behavior in *Hydra*. Pages 369–390 in W. C. Corning and S. C. Ratner, Eds., *Chemistry of Learning*. Plenum Press, New York.

RUSHFORTH, N. B., 1967b. Behavioral responses of isolated tentacles of *Hydra*. *Biol. Bull.*, **133**: 449.

SHIBLEY, G. A., 1969. Gastrodermal contractions correlated with rhythmic potentials and prelocomotor bursts in *Hydra*. *Amer. Zool.*, **9**: 586.

STRUMWASSER, F., 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. Pages 442–462 in J. Aschoff, Ed., *Circadian Clocks*. North Holland Publishing Co., Amsterdam.

STRUMWASSER, F., 1967. Types of information stored in a single neuron. Pages 291–319 in C. A. G. Wiersma, Eds., *Invertebrate Nervous Systems*. University of Chicago Press, Chicago, Illinois.

Reference: *Biol. Bull.*, **140**: 274–283. (April, 1971)

REGULATION OF GONAD DEVELOPMENT IN THE BAY SCALLOP, *AEQUIPECTEN IRRADIANS* LAMARCK[1]

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Gonad development in the annual reproductive cycle of temperate climatic zone marine invertebrates is correlated with seasonal changes in the environment. Gonad development has been induced in molluscs (Loosanoff and Davis, 1950; Sastry, 1963) and barnacles (Crisp, 1957; Patel and Crisp, 1960) outside the normal breeding period by maintaining the animals at the appropriate temperatures and providing food. The effect of photoperiod on maturation and breeding of *Balanus* has been reported by Barnes (1963). The influence of food on gonad development in sea urchins and scallops has been reported by Giese (1959) and Sastry (1966). Barnes (1967) found that interruption of food supply leads to regression of ovarian tissue in barnacles, especially in the early stages of development. Although environmental influence on gonad growth and gametogenesis is apparent, the mechanism of control is still unclear.

Sastry (1968) reported that gonad growth and gametogenesis are initiated in bay scallops, *Aequipecten irradians* Lamarck, when the animals are exposed to a minimum threshold temperature with food. In the absence of food or when the temperature remains below the threshold level, the oogonia develop but oocyte growth does not occur. This suggests that nutrient reserves from the ingested food are utilized during gonad growth and gametogenesis. The nutrient reserves are apparently transferred from the storage organ, the digestive gland, to the gonad and utilized by the developing gametes for synthesis of various biochemical constituents.

The present paper considers the influence of temperature on the transfer of nutrient reserves.

MATERIALS AND METHODS

Source and maintenance of animals

Bay scallops collected during the last week of November from Buzzards Bay, Massachusetts (10° C and 32‰), were obtained from the Supply Department, Marine Biological Laboratory, Woods Hole. The scallops were maintained in the laboratory at 5° C and 15° C in recirculating sea water aquaria (Dayno Aqua-Labs, Inc., Lynn, Massachusetts) with a photoperiod of 12 hours light and 12 hours darkness. Scallops were fed daily on naked flagellates, *Monochrysis lutheri* Droop cultured in the laboratory on an enriched sea water medium described by Davis and Guillard (1958). Three liters of food organisms (2–3 million cells/ml) were provided daily to a group of 80 scallops held at each experimental temperature.

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Assay of reproductive activity

At the beginning of the experiments, ten scallops were sacrificed, and the gonad index (gonad weight/body weight × 100), digestive gland index (digestive gland weight/body weight × 100), and the stage in gametogenesis were determined (Sastry, 1966). Five animals were removed at intervals from each experimental temperature and the same measurements made. The oocyte diameter in each animal from each sample was measured with an ocular micrometer.

Radiotracer experiments

Radiotracer experiments were performed by injecting uniformly labelled ^{14}C-leucine (New England Nuclear Corp., Boston, Massachusetts) into the digestive gland of the scallops. Radioactive leucine (specific activity of 240 MC/m*M* dissolved in 0.5 ml. 0.01 *N* HCl was added to 0.5 ml of sea water (32‰) to make an approximately isotonic solution. Ten μl (0.5 μC) of solution per animal was injected into scallops held at 5° C and 15° C on the 15th, 30th and 45th day after the beginning of the experiment. The scallop injected with the labelled amino acid were placed in battery jars containing sea water of equilibrated temperature and kept in contsant temperature incubators. Each scallop was fed 100 ml of food organisms a day. Four or five animals were sacrificed after one day and again after one week to determine the gonad and digestive gland indexes and the stage in gametogenesis. Pieces of body tissues weighing 15–20 mg taken from each animal were dissolved in 1 ml of Soluene-100 (Packard Instrument Co.) in low potassium vials. The vials were kept for at least 18 hours at 35° C to aid in dissolving the tissue. The vials were then filled with Bray's solution (Bray, 1960). The scintillation mixture contained 60 g naphthalene, 4 g PPO, 500 ml POPOP, 100 ml methanol, 20 ml of ethylene glycol in 880 ml of p-dioxine to make up to

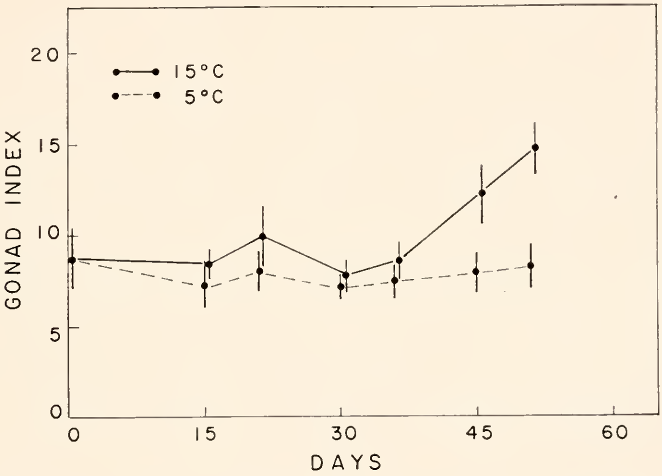

FIGURE 1. Gonad growth response of scallops at 5° C and 15° C.

a total volume of 1 liter. Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter (model 3380) attached to a computer print out. Appropriate corrections were made for background and quenching.

The total disintegrations per minute (DPM) for organs of each animal were calculated by the following method:

$$\text{Total DPM/organ} = \text{DPM/mg tissue} \times \text{total weight of organ (mg)}.$$

The DPM for all body organs were added to obtain the total DPM per animal. Incorporation of labelled amino acid into the organs is calculated by the following method:

$$\text{Radioactivity index} = (\text{Total DPM/organ} \times 100/\text{Total DPM/animal})$$

Statistical analysis of the data was made with the aid of IBM-360 computer.

RESULTS

Gonad growth response of scallops at 5° C and 15° C

The mean gonad index values of scallops held at 5° C and 15° C are shown in Figure 1. The gonad index remained approximately the same for all samples from 5° C The scallops at 15° C showed an increase of gonad index following day 30. The differences between the gonad indexes of samples kept at the two temperatures became significant by day 45.

The gonad and digestive gland indexes of scallops show a reciprocal relationship during the reproductive cycle (Sastry, 1970a). The digestive gland index, greater than the gonad index during the vegetative and resting periods, decreases with the gonad growth. This decrease has been attributed to the transfer of nutrients to the gonads (Sastry, 1966, 1968). The relationship between the two organ indexes of scallops exposed to 5° C and 15° C is shown in Figure 2 and Table I. The digestive gland index remained near a constant level above the gonad index for scallops at 5° C. At 15° C, however, the digestive gland index fluctuated, and showed an increase with the growth of gonads. Apparently the clear reciprocal relationship observed for field population does not apply to animals held in the laboratory with abundant food.

Gametogenesis in scallops at 5° C and 15° C

The scallops examined at the beginning of the experimental period had only primary germ cells. The oocyte growth in scallops held at 5° C and 15° C is shown in Figure 3. Oogonia developed at 5° C but there was no oocyte development. The scallops held at 15° C developed oocytes in the cytoplasmic growth phase by day 21 and the vitellogenesis growth phase by day 36. Most oocytes were in the vitellogenesis growth phase on day 51.

Spermatocytes and spermatids were observed in the scallops at the beginning of the experiment. In the scallops held at 5° C spermatocytes were the dominant stage throughout the experimental period, but a few spermatozoa also were observed. At 15° C spermatozoa were predominant, but spermatocytes and spermatids were present.

Incorporation of ^{14}C-leucine into the digestive gland and gonad of scallops at 5° C and 15° C, 24 hours after injection

The mean radioactivity index for ^{14}C-leucine incorporation into the digestive gland and the gonad of scallops at 5° C and 15° C is given in Figure 4 and Table II. The animals injected with the labelled amino acid on day 15 and 30 at 5° C showed a wide variation of incorporation into the digestive gland. The mean radioactivity index for labelled amino acid incorporation was slightly higher in the sample injected on day 30. Incorporation of ^{14}C-leucine into gonads was much less than into the digestive gland in all the samples. The gonads of samples injected on days 30 and 45 took up slightly more than those injected on day 15.

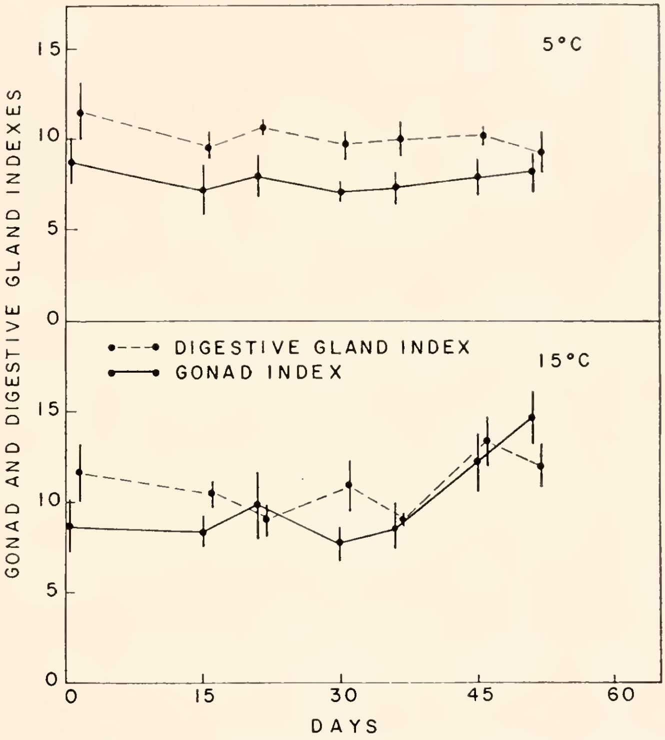

FIGURE 2. Mean gonad index and digestive gland index of scallops exposed to temperatures of 5° C and 15° C.

In the scallops held at 15° C the incorporation of ^{14}C-leucine into the digestive gland of all samples was about the same except for day 30, when it was slightly higher. Again the gonads took up much less amino acid than the digestive gland. The mean radioactivity index for incorporation of ^{14}C-leucine into the gonads was about the same in samples injected at different time intervals.

Incorporation of ^{14}C-leucine into both digestive gland and gonad of scallops held at 15° C was higher than in the animals at 5° C (Table II).

Incorporation of ^{14}C-leucine into digestive gland and gonad of scallops at 5° C and 15° C, one week after injection

The incorporation of ^{14}C-leucine into the digestive gland and gonad of scallops at 5° C and 15° C is shown in Figure 4 and Table III. Uptake of labelled amino acid by the digestive gland was about the same on day 15 and day 45 for scallops held at 5° C. The incorporation of ^{14}C-leucine into the gonad, while much lower than that for the digestive gland, was slightly higher on day 45 than on day 15.

In the scallops held at 15° C, the mean radioactivity index for incorporation of labelled amino acid into the digestive gland was about the same in all the samples. At this temperature, however, the gonads incorporated more ^{14}C-leucine than the digestive gland on days 30 and 45.

TABLE I

Mean gonad and digestive gland indexes of scallops at 5° C and 15° C

Day	Organ	5° C	15° C	Significance (t-test) t
0	G	8.7 ± 1.4	8.7 ± 1.4	—
	D	11.6 ± 1.6	11.6 ± 1.6	—
15	G	7.3 ± 1.3	8.4 ± 0.8	1.58
	D	9.6 ± 0.8	10.4 ± 0.7	1.74
21	G	8.0 ± 1.1	9.8 ± 1.8	1.87
	D	10.7 ± 0.3	9.0 ± 0.8	4.41*
30	G	7.2 ± 0.6	7.7 ± 0.9	1.08
	D	9.6 ± 0.8	10.9 ± 1.4	1.08
36	G	7.4 ± 0.9	8.6 ± 1.1	2.10
	D	10.1 ± 0.8	9.0 ± 0.1	1.95
45	G	7.9 ± 1.0	12.2 ± 1.6	5.05*
	D	10.3 ± 0.4	13.4 ± 1.4	4.64*
51	G	8.3 ± 1.1	14.7 ± 1.4	8.13*
	D	9.4 ± 1.1	12.1 ± 1.2	3.91*

G, Gonad; D, Digestive gland; * $P \leq 0.05$.

The difference in the uptake of ^{14}C-leucine into the gonads of scallops held at 5° C and 15° C is statistically significant (Table III). Incorporation of ^{14}C-leucine into the digestive gland of scallops held at 15° C was higher than in the animals held at 5° C. The difference is only significant in the samples injected on day 45 ($P < 0.05$).

DISCUSSION

The influence of environmental factors on the gonad development of marine invertebrates has been demonstrated, but the mechanisms controlling gonad growth in the reproductive cycle are not yet clear. Breeding condition has been induced in

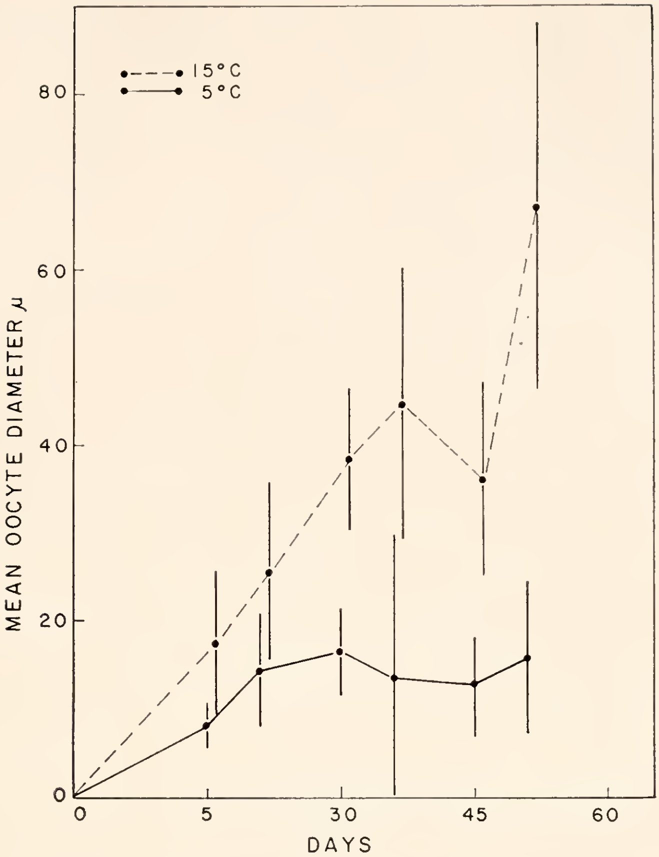

FIGURE 3. Oocyte growth in scallops at 5° C and 15° C.

TABLE II

Incorporation of ^{14}C-leucine into gonad and digestive gland of scallops at 5° C and 15° C, 24 hours after injection

Day of injection	Organ	5° C Radioactivity index	15° C Radioactivity index	Significance (t-test) t
15	G	3.8 ± 2.3	11.1 ± 7.8	1.99
	D	13.6 ± 10.4	18.9 ± 4.4	0.96
30	G	6.8 ± 3.8	7.3 ± 3.4	0.23
	D	20.7 ± 17.7	26.7 ± 10.0	0.66
45	G	7.0 ± 2.0	9.8 ± 1.4	2.51*
	D	14.0 ± 2.5	19.1 ± 2.5	3.00*

G, Gonad; D, digestive gland; * $P \leq 0.05$.

bivalve molluscs and barnacles by maintaining the animals at appropriate temperatures in the winter and supplying food. This suggests a temperature and nutritional influence on gonad development (Loosanoff and Davis, 1950; Patel and Crisp, 1960; Sastry, 1963). Crisp (1957) reported that *Balanus* required maintenance at colder temperatures for a prolonged period before they could be induced to breed at warm temperatures. However, exposure to warm temperatures alone failed to induce breeding condition in *Balanus*. Starvation of a number of species at the beginning of the reproductive period was reported to prevent gonad growth which suggests that food influences gonad development (Giese, 1959; Sastry, 1966; Barnes, 1967). Apparently, the nutrient reserves from the ingested food must be transferred to gonads for growth to occur and these materials are utilized there in the biochemical synthesis of the developing gametes (Giese, 1959; Barnes, Barnes and Finlayson, 1963). It has been suggested that nutrient reserves are transferred from the storage organ in barnacles and starfish or directly from gut to perivisceral fluid and then to gonads in sea urchins without significant storage (Giese, 1959;

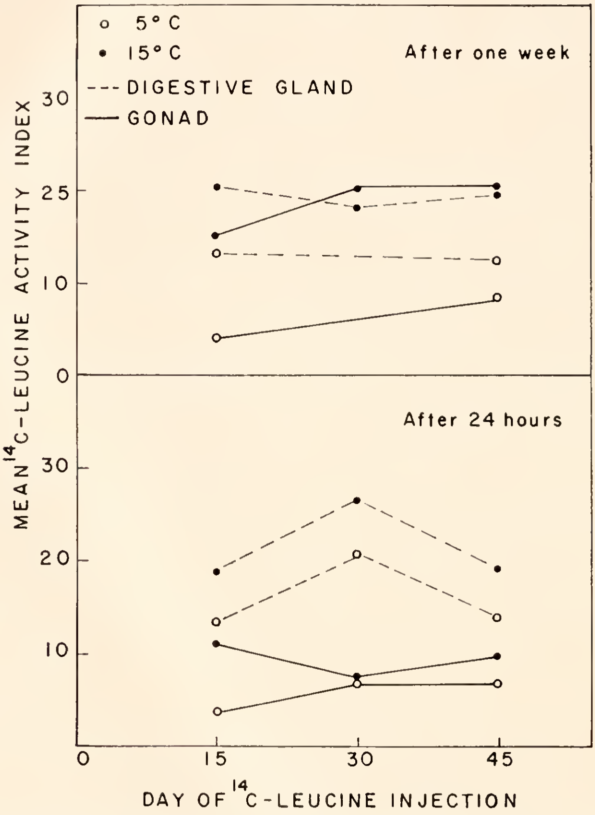

FIGURE 4. Incorporation of ^{14}C-leucine into gonad and digestive gland of scallops, 24 hours after injection and one week after injection.

Farmanfarmaian and Phillips, 1962; Barnes, Barnes and Finlayson, 1963; Mauzey, 1966). The transfer of nutrient reserves to the gonads is thought to occur with the initiation of gonad development by establishment of a gradient between source and utilization (Barnes, Barnes and Finlayson, 1963). However, there are only a few studies examining the distribution and utilization of nutrients during reproduction in marine invertebrates. Allen (1962) found that *Mya arenaria* fed with ^{32}P labelled *Pheodactylum* assimilates greatest concentrations of ^{32}P in the digestive gland with little evidence of rapid build up in the ovary. In *Calanus,* however, Marshall and Orr (1955, 1961) found that 70% of the ^{32}P assimilated by the female copepod passes rapidly into the ova. The results of the present study show that transfer of nutrient reserves to gonads occurs in scallops with the initiation of gonad development. Gonad development is initiated in fed animals maintained within a range of temperatures (Sastry, 1968). Under these conditions, the nutrient reserves are apparently transferred to the gonads. Temperature below the required minimum supresses gonad growth and the nutrient reserves are accumulated in the digestive gland. The nutrient reserves stored in the digestive gland may supplement the nutritional intake needed for gonad growth. However, the animals maintained at temperatures exceeding a certain maximum also fail to develop gonads. Perhaps this is due to increased metabolic utilization or failure to regulate the synthetic processes of the developing gametes.

Table III

Incorporation of ^{14}C-leucine into gonad and digestive gland of scallops at 5° C and 15° C one week after injection

Day of injection	Organ	5° C Radioactivity index	15° C Radioactivity index	Significance (t-test) t
15	G	4.2 ± 1.1	15.7 ± 7.8	3.12*
	D	13.2 ± 12.9	20.6 ± 11.8	0.96
30	G	—	20.0 ± 12.0	—
	D	—	18.2 ± 1.8	—
45	G	8.5 ± 2.2	20.4 ± 3.4	6.54*
	D	12.4 ± 3.4	19.8 ± 3.0	3.48*

G, Gonad; D, Digestive gland; * $P \leq 0.05$.

Gametogenesis occurs simultaneously with gonad growth in scallops. Oocytes enter the cytoplasmic growth phase when the animals are exposed to the required minimum temperature with food. Either subthreshold temperature or starvation will prevent the oocytes from developing (Sastry, 1968). Apparently initiation of the cytoplasmic growth phase is regulated by the transfer of nutrients to the gonads, this transfer, of course, being dependent on the temperature. These nutrients are obviously utilized by the oocytes during their growth to mature eggs. The nutrient transfer to the gonads is therefore not only regulated by environmental factors, but also by the nutritional needs of the developing oocytes.

The significant increase in the incorporation of ^{14}C-leucine into gonads of scallops with oocytes in the cytoplasmic growth phase at 15° C further suggests that the rate of nutrient transport to the gonads is regulated by the temperature and the stage of oocyte development (Figs. 3 and 4). However, the uptake of labelled amino acid into gonads of scallops at 5° C and 15° C measured 24 hours after injection is not significantly different. The nutrient transfer may occur slowly at a rate regulated by the developing oocytes.

The difference in the incorporation of ^{14}C-leucine into the gonads of scallops at 5° C and 15° C could have been influenced by the temperature, or the stage of oocyte development, or both. Incorporation of ^{14}C-leucine into gonads of scallops with oogonia at 5° C is much less than those with oocytes in the cytoplasmic growth phase at 15° C, especially one week after injection. Incorporation of ^{14}C-leucine has been shown to be involved in protein synthesis (Raven, 1961; Williams, 1965). It has also been reported that protein synthesis occurs in growing medium-sized oocytes but is absent in very young or mature unfertilized eggs of the sea urchin. It is possible that oocytes entering the growth phase stimulate gonad development, with the rate of gonad development depending on the rate at which nutrient reserves are utilized by the growing oocytes for synthesis of various biochemical constituents. Oocytes entering the cytoplasmic growth phase may begin protein synthesis by utilizing the nutrients taken up from the digestive gland. The cytoplasmic growth phase of oocytes may be triggered either by direct action of temperature or by neurosecretion or both (Lubet, 1966; Sastry, 1970b).

It is a pleasure to thank Dr. Eugene Jackim and Dr. Earl Davey, National Marine Water Quality Laboratory, West Kingston, for the use of facilities for radioactivity measurements and for assistance during this study. Thanks are also due to Miss Katherine Saunders for technical assistance.

SUMMARY

1. Gonad growth and oocyte cytoplasmic growth were induced in scallops exposed to 15° C with food. At 5° C only oogonia developed.

2. Scallops injected with ^{14}C-leucine and measured 24 hours after injection showed greater incorporation of labelled amino acid into both digestive gland and gonad at 15° C than at 5° C, but the difference in uptake between the two groups was not significant.

3. Incorporation of ^{14}C-leucine into gonads of scallops measured one week after injection was significantly higher at 15° C than at 5° C.

4. The gonad growth and oocyte growth of scallops is controlled by food and temperature. Both external environmental factors and the stage of oocyte development seem to regulate nutrient transfer to gonads and therefore control growth activity.

LITERATURE CITED

Allen, J. A., 1962. Preliminary experiments on the feeding and excretion of bivalves using *Phaeodactylum* labelled with ^{32}P. *J. Mar. Biol. Ass. U. K.*, **42**: 609–623.

Barnes, H., 1963. Light, temperature and breeding of *Balanus balanoides*. *J. Mar. Biol. Ass. U. K.*, **43**: 717–728.

Barnes, H., 1967. The effect of starvation and feeding on the time of production of egg masses in the boreo-arctic Cirripede, *Balanus balanoides* (L.). *J. Exp. Mar. Biol. Ecol.*, **1**: 1–6.

Barnes, H., M. Barnes and M. Finlayson, 1963. The seasonal changes in body weight, biochemical composition and oxygen uptake of two common boreo-arctic barnacles, *Balanus balanoides* and *B. balanus*. *J. Mar. Biol. Ass. U. K.*, **43**: 185–212.

Bray, George A., 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.*, **1**: 279–288.

Crisp, D. J., 1957. Effect of low temperature on the breeding of marine animals. *Nature*, **179**: 1138–1139.

Davis, H. C., and R. R. Guillard, 1958. Relative value of ten genera of microorganisms as food for oysters and clam larvae. *Fish. Bull. U. S. Fish Wildlife Serv.*, **58**: 293–304.

Farmanfarmaian, A., and J. H. Phillips, 1962. Digestion, storage, and translocation of nutrients in the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.*, **123**: 105–120.

Giese, A. C., 1959. Comparative physiology: Annual reproductive cycle of marine invertebrates. *Annu. Rev. Physiol.*, **21**: 547–576.

Loosanoff, V. L., and H. C. Davis, 1950. Conditioning of *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull.*, **98**: 60–65.

Lubet, P., 1966. Essai d'analyse experimentale des perturbations produites par les abaletions des ganglions nerveux Chez *Mytilus edulis* L and *Mytilus galloprovincialis* LMK (Mollusques Lamellibranches). *Ann. Endoc.* **27**: 353–365.

Mauzey, K. P., 1966. Feeding behavior and reproductive cycles in *Pisaster ochraceus*. *Biol. Bull.*, **131**: 127–144.

Marshall, S. M., and A. P. Orr, 1955. On the biology of *Calanus finmarchicus*. VII. Food uptake, assimilation and excretion in adult and stage *V. Calanus*. *J. Mar. Biol. Ass. U. K.*, **34**: 495–529.

Marshall, S. M., and A. P. Orr, 1961. Studies on the biology of *Calanus finmarchicus*. XII. The phosphorous cycle: excretion, egg production and autolysis. *J. Mar. Biol. Ass. U. K.*, **41**: 463–488.

Patel, B., and D. J. Crisp, 1960. The influence of temperature on the breeding and moulting activities of some warm water operculate barnacles. *J. Mar. Biol. Ass. U. K.*, **39**: 667–680.

Raven, Chr. P., 1961. *Oogenesis: The Storage of Development Information.* Pergamon Press, New York, 274 pp.

Sastry, A. N., 1963. Reproduction of the bay scallop, *Aequipecten irradians* Lamarck. Influence of temperature on maturation and spawning. *Biol. Bull.*, **125**: 146–153.

Sastry, A. N., 1966. Temperature effects in reproduction of the bay scallop, *Aequipecten irradians* Lamarck. *Biol. Bull.*, **130**: 118–134.

Sastry, A. N., 1968. The relationships among food, temperature and gonad development of the bay scallop, *Aequipecten irradians* Lamarck. *Physiol. Zool.*, **41**: 44–53.

Sastry, A. N., 1970a. Reproductive physiological variation in latitudinally separated populations of the bay scallop, *Aequipecten irradians* Lamarck. *Biol. Bull.*, **138**: 56–65.

Sastry, A. N., 1970b. Environmental regulation of oocyte growth in the bay scallop, *Aequipecten irradians* Lamarck. *Experentia*, in press.

Williams, J., 1965. Chemical constitution and metabolic activities of animal eggs. Pages 13–71 in Rudolf Weber, Ed., *The Biochemistry of Animal Development, Descriptive Biochemistry of Animal Development, Volume 1.* Academic Press, New York.

Reference: *Biol. Bull.*, **140**: 284–322. (April, 1971)

LARVAL DISPERSAL AS A MEANS OF GENETIC EXCHANGE BETWEEN GEOGRAPHICALLY SEPARATED POPULATIONS OF SHALLOW-WATER BENTHIC MARINE GASTROPODS[1]

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If the larvae of marine gastropods are capable of frequent long-distance dispersal, then they can be expected to serve, not only as a means of colonizing or re-colonizing new regions, but also as agents of gene flow between widely separated or disjunct populations. Populations that become completely isolated will tend to evolve in different directions. On the other hand, frequent genetic exchange between populations of the same species will counteract the divergent evolution between them, particularly if the immigrant veligers introduce especially advantageous genes upon which selection may act.

To support the hypothesis that larvae are important in maintaining genetic continuity between shoal-water populations of gastropods with amphi-Atlantic distributions, it is necessary first to give evidence that larvae are indeed dispersed over long distances and second to show that the frequency of larval dispersal is related to converging or diverging evolution of isolated adult populations of gastropod species.

Two kinds of evidence are useful in demonstrating long-distance dispersal: (1) the relationship between the circulation and the geographical distribution of larvae and (2) the duration of pelagic larval life and its relationship to the velocity of trans-Atlantic currents. Thus, larvae should be present along the entire length of the surface currents required to transport them between the geographically disjunct adult populations, and the length of pelagic larval life must be long enough so that currents will carry veligers the required distance within the time allotted them.

To understand the frequency of larval dispersal it is necessary to know: (1) the probability that larvae will survive and be transported by off-shore currents to far-distant populations and (2) the approximate number of larvae produced by the originating or parent population.

In this account I will attempt to show that larvae are dispersed over long distances, that the frequency of dispersal is much higher than might superficially be supposed, and finally, in a preliminary way, to show that the relative frequency of larval dispersal does indeed seem to be related to morphological similarities or differences found between spatially separated populations.

METHODS

The conclusions presented here are based on 857 plankton tows taken throughout the tropical and warm-temperate North Atlantic Ocean (Fig. 4). The samples were obtained from the research vessels ATLANTIS II and CHAIN by making oblique tows with a three-quarter-meter plankton net having a mesh of 223 or 316 microns. In most instances a net with the smaller mesh was used. The samples were taken by paying out 200 meters of wire at approximately 20 meters per minute and then hauling the net inboard at a similar rate. The duration of a tow was 20 minutes and the ship moved at approximately 5.5 km/hr (3 knots). The maximum depth to which the net descended depended on current and weather conditions, but was usually about 100 meters.

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[1] Contribution No. 2565 from the Woods Hole Oceanographic Institution.

Preliminary sorting was done at sea where living larvae were separated from the sample. Observations of live veligers proved to be quite valuable for later identification. Certain species were selected for rearing to settlement; these were maintained in one-liter polyethylene boxes and fed from phytoplankton cultures maintained at sea. In addition to tows made by me from the research vessels of the Woods Hole Oceanographic Institution, I have also had access to a number of other plankton samples loaned to me by various colleagues.

Very few previous taxonomic investigations have been made on tropical gastropod larvae (*vide* Simroth, 1895; Thorson, 1940; Dawydoff, 1940; Lebour, 1945), so that the adult affinities of each veliger form must be determined. Identifications can be made either by (1) comparing the larval shell with the protoconch of an adult and (2) rearing the larvae in the laboratory through settlement and post-larval development.

In practice, at least with tropical species, the latter method presents considerable difficulties. Though I have held some species of veliger larvae to settlement in the laboratory (*viz.*, *Cymatium nicobaricum, Thais haemastoma*, and *Philippia krebsii*), generally it is not readily possible to maintain the post-larvae to a stage at which identification becomes possible, because the feeding requirements of most tropical gastropods are still quite unknown.

Identifications of the gastropod veligers considered here were made by careful comparison between larval shells and protoconchs of young adult specimens. I am indebted to the curators of molluscs, Drs. J. Rosewater, U. S. National Museum, R. T. Abbott and R. Robertson, Academy of Natural Sciences of Philadephia, and W. J. Clench and K. J. Boss, Museum of Comparative Zoology, Harvard University, for allowing me the use of collections under their custody. Mr. H. Lewis of the Philadelphia Shell Club kindly made specimens of Cymatiidae available to me. Usable specimens having protoconchs in good condition are relatively rare for most species. In identifying any species of larva, I have looked at the protoconch of specimens of all other closely related species that occur in the tropical and warm-temperate Atlantic if the material were available.

Results and Discussion

Evidence for long-distance larval dispersal

The relationship between circulation and geographical distribution of larvae

The importance of larval dispersal was already recognized by Alfred Russel Wallace in his work on *The Geographical Distribution of Animals* (1876). Wallace knew that the univalve and bivalve Mollusca have free-swimming larval stages and recognized that "they thus have a powerful means of dispersal, and are carried by tides and currents so as ultimately to spread over every shore and shoal that offers conditions favorable for development." (Vol. I, page 30). Simroth (1895) described and figured pelagic Mollusca, including gastropod veliger larvae, from samples taken on the *Plankton Expedition*, though he did not establish the identity of most of the forms he described. Thorson (1961, page 466) recognized that certain genera of gastropods, principally tropical in their geographical distribution (*e.g., Lamellaria, Tonna, Cassis, Charonia, Cymatium,* and *Bursa*), have larvae that "have been found midway across the oceans." Recently, Robertson (1964) and Scheltema (1964, 1966a) have described the dispersal of two species of gastropods into the Sargasso Sea and Gulf Stream, while Mileikovsky (1966) has reviewed existing knowledge on the dispersal of molluscan larvae, particularly the dispersal of prosobranch veligers in the Norwegian Sea. The gastropod families that are now known to have representatives with long pelagic developments include the families Neritidae and Naticidae (first mentioned here), and the Triphoridae, Lamellariidae, Architectonicidae, Cymatiidae, Cassidae, Tonnidae, Muricidae, Bursidae, Coralliophilidae, Ovulidae, and Cypraeidae (Scheltema, 1966b).

While scattered records have already shown the existence of larvae in the open sea, no systematic attempt has yet been made to determine their occurrence over large areas of the ocean and in particular their presence throughout the entire length of major current systems of the equatorial and north temperate Atlantic Ocean. These data are the most important in demonstrating the dispersal of larvae over long distances.

The evidence from plankton collections I have made shows that dispersal of gastropod larvae into the open sea is not a rare event but a commonplace occurrence (Fig. 4). Taking into account all collections examined, including samples from all major currents of warm waters of the North Atlantic Ocean, 70 per cent contain gastropod larvae from the shoal-water benthos. Many species are found during all months of the year. Because of their common occurrence and in order to avoid the cumbersome expression "long-distance larvae from shoal-water bottom-dwelling organisms of the shelf," I have applied to these open-sea forms the term *teleplanic larvae* or *teleplanos* (from the Greek *telep'lanos,* meaning far-wandering) (Scheltema, in press). The teleplanos may be defined as larvae that (1) originate from shoal-water, continental-shelf benthos, (2) are regularly found in the open sea, (3) have a pelagic development of long duration, and (4) serve as a means for dispersal over long distances. The term is not restricted taxonomically to any phylum, but specifically excludes larvae of the holoplankton. Many teleplanic gastropod veligers have morphological adaptations which enhance their survival on the open ocean, such as long periostracal spines, the reduction or complete lack of shell calcification, and an increase in length of the velar lobes used for swimming and feeding.

The North Atlantic circulation. The direction and route of larval dispersal are determined by the principal ocean currents. The circulation of the North Atlantic Ocean may be regarded as an enormous anticyclonic gyre. On the west is the Gulf Stream moving northeastwardly; on the north is the North Atlantic Drift moving toward the east and dividing into a northern and southern arm; bounding the eastern portion of the gyre is the Canary Current moving to the southwest; and finally on the south is the North Equatorial Current moving westwardly across the tropical North Atlantic toward the West Indies (Fig. 4). Details of this circulation are obviously much more complex. Large temporary eddies are known to occur in the Gulf Stream (Fuglister, 1963) and the North Atlantic Drift is grossly over-simplified by this description (*vide* Worthington, 1962; Mann, 1967). Notwithstanding, this generalized conception allows prediction of the transport expected from an object floating on or near the ocean surface. In the Equatorial Atlantic Ocean the North Equatorial Current is separated from the westwardly flowing South Equatorial Current by a weakly developed Equatorial Countercurrent flowing toward West Africa. The Countercurrent moves only a relatively small volume of water and is seasonal in occurrence (Schumacher, 1940, 1943; *U. S. Hydrographic Office Monthly Pilot Charts*). However, directly beneath the westwardly flowing South Equatorial Current a strong eastwardly moving Equatorial Undercurrent flows from South America toward the island of São Tomé off West Africa (Voorhis, 1961; Voigt, 1961; Metcalf, Voorhis and Stalcup, 1962; Khanaychenko, Khlystov and Zhidov, 1965; Rinkel, Sund and Neuman, 1966; Strum and Voigt, 1966). This warm, shallow current (20° to 27° C), whose core extends between 50 and 100 meters depth, provides a means whereby tropical larvae may be dispersed from west to east across the tropical Atlantic (Chesher, 1966, page 210; Scheltema, 1968).

Description of larvae and their geographical distribution in the North and Equatorial Atlantic Ocean. To illustrate specific instances of teleplanic gastropod larvae in temperate and tropical Atlantic waters, I have selected to consider in detail, from among many species, ten representative forms taken from the families Cymatiidae, Tonnidae, Cassidae, Muricidae, Architectonicidae, and Neritidae. Since the larvae of these ten species are either completely unknown or not previously well described, each is illustrated and briefly described here.

Family Cymatiidae

The family Cymatiidae or hairy tritons is a group of carnivorous gastropods which are very well represented in the tropical Atlantic. The three species considered here, *Cymatium parthenopeum* (von Salis), *Cymatium nicobaricum* (Röding), and *Charonia variegata* (Lamarck), all have very wide geographical distributions. *Cymatium parthenopeum* is known throughout most of the warm and tropical North and South Atlantic and as *Monoplex australasiae* Perry in the Indo-Pacific (Clench and Turner, 1957). *Cymatium nicobaricum* is common in the western Atlantic and Indo-Pacific but has also been reported from a few records in the eastern Atlantic (Dautzenberg, 1890; Odhner, 1931; Clench and turner, 1957). The egg masses of several Cymatiidae are known from recent studies of Laxton (1969).

The larva of *Cymatium parthenopeum* has an uncalcified, resilient, translucent, amber-colored, conical, multispiral shell which becomes somewhat darker in color when nearing completion of planktonic development (Fig. 1, d, e, f). The first whorl following the embryonic shell is cancellate; the remaining whorls are glossy and smooth (Fig. 1, j). The shell in early stages is holostomatous, becoming siphonostomatous with a short siphonal canal when reaching the intermediate stage (Fig. 1, d; see also Scheltema, 1966a, Figs. 4a and 4b). The outer lip has a varix when larval growth is completed (Fig. 1, e, f). The slightly impressed suture of the body whorl is mostly obscured by the smooth, overlying

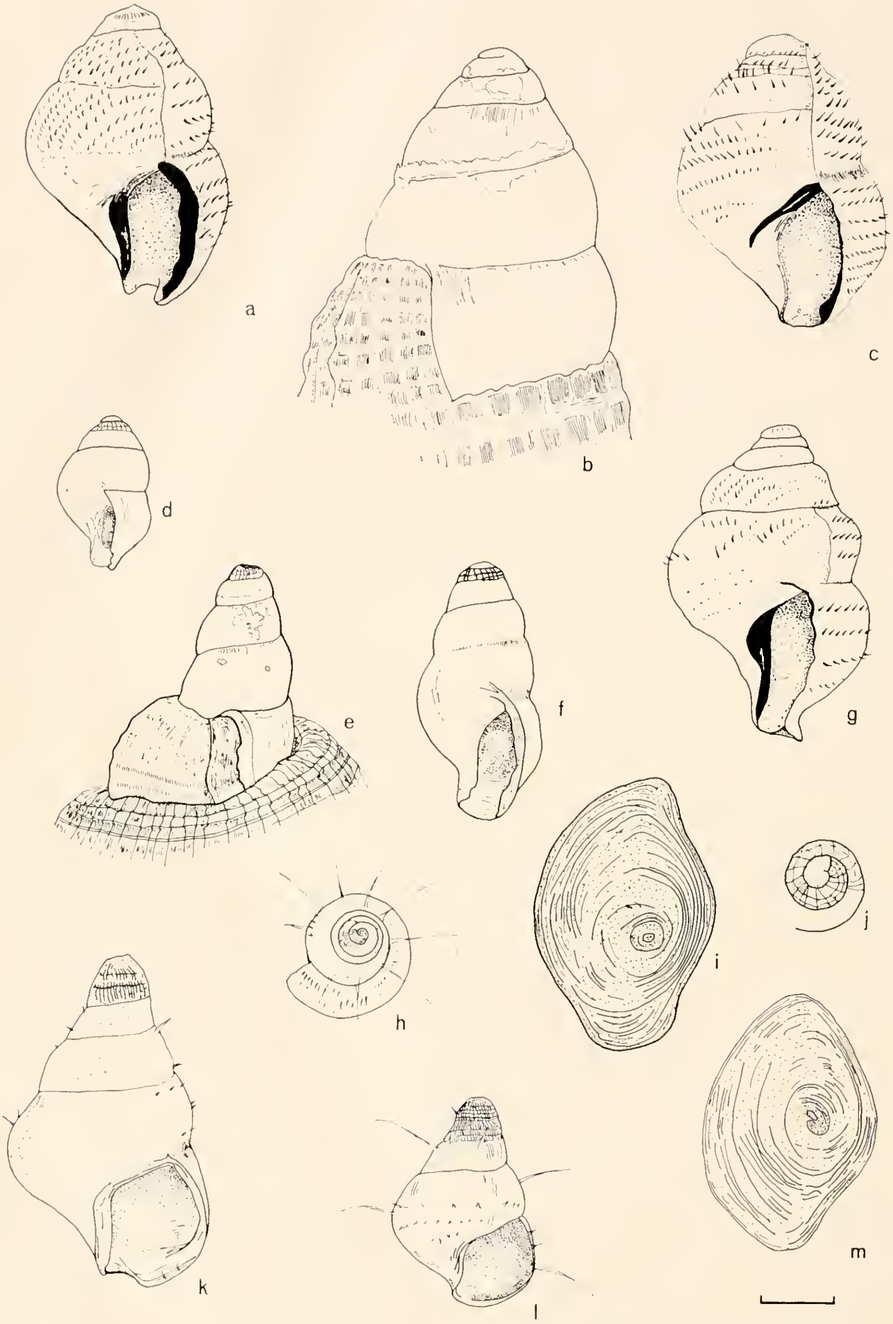
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FIGURE 1.

periostracum. The operculum shows practically no markings; a very faint eccentric nucleus is sometimes visible. In the living larva the body is light cream-colored, while the foot is gray to black. The larva has two tentacles and four long velar lobes lacking pigmentation (*vide* Scheltema, 1966a, Fig. 2). When growth is completed the larval shell is about 3.5 mm in length. The unknown larval shell figured by Simroth (1895, Table V, Fig. 16) is probably this species. The teleplanic larvae of *Cymatium parthenopeum* are found throughout the North Atlantic gyre, in the Caribbean, and from two locations in the western end of the south Equatorial Current (Fig. 5).

The larva of *Cymatium nicobaricum* was described by Lebour (1945, pages 477–478, Fig. 24, as *Cymatium chlorostomum*) from the waters near Bermuda, and the species was also figured but unidentified by Simroth (1895, Pl. V, Figs. 10 and 11). It is very easily recognized because it is the only *Cymatium* larva in the Atlantic having a coeloconoid shell (Fig. 1, k, l). The first 1½ whorls following the embryonic shell are cancellate. On the completely developed larva the embryonic and sometimes a portion of the cancellate whorls are already worn. The early and intermediate stage larvae have long, slightly curved, periostracal spines (Fig. 1, h, l) which in older larvae may no longer be present (Fig. 1, k). Minute axial lines are sometimes barely visible in the periostracum. The operculum (Fig. 1, m) differs from most Cymatiidae in that its nucleus is only very slightly eccentric. Only *Charonia variegata* has a similar operculum (Fig. 1, i), but the larvae of *Cymatium nicobaricum* cannot be confused with this species. In the living larva of *Cymatium nicobaricum* the body pigmentation is pink with darker reddish patches. There are four long velar lobes which completely lack pigmentation. The length of the fully grown larval shell is about 4.6 mm. It is very often encrusted with fouling organisms including Folliculinidae, green algae, and encrusting bryozoa. *Cymatium nicobaricum* larvae are restricted in the samples to the western Atlantic and North Atlantic Drift (Fig. 5) but were never found in the eastern Atlantic.

Charonia variegata has the largest known larvae of any of the Cymatiidae in the Atlantic, the shell exceeding 5 mm in length when fully developed. Its ovate,

FIGURE 1. Larval shells, opercula and protoconchs of three species of Cymatiidae: (a) larval shell of *Charonia variegata*, Northwestern Atlantic, 39°27′N, 50°17′W; (b) protoconch of *Charonia variegata*, Western Atlantic, Tobago Island, B.W.I. (ANSP 209812); (c) *Charonia variegata* larval shell, North Equatorial Current, 19°11′N, 47°24′W; (d) intermediate stage veliger larva of *Cymatium parthenopeum*, Gulf Stream, 38°43′N, 70°46′W; (e) protoconch of *Cymatium parthenopeum*, collection of H. Lewis (No. 2493); (f) late veliger larva of *Cymatium parthenopeum* at final stage before settling, off the Azores, 36°32′N, 30°14′W; (g) larva of *Charonia variegata*, Eastern Atlantic off the West Coast of Africa, *Atlantide* Station No. 122, 01°29′S, 08°50′E; (h) apical view of intermediate stage larva of *Cymatium nicobaricum*, Gulf Stream, 35°44′N, 66°53′W; (i) operculum from larva of *Charonia variegata*, internal view, specimen from same station as (a) above; (j) embryonic shell and first cancellate whorl of the pelagic larva of *Cymatium parthenopeum*, Gulf Stream, same station as (d) above; (k) late veliger larva of *Cymatium nicobaricum* at final stage before settlement, Western Atlantic near Bermuda, 32°45′N, 64°36′W; (l) intermediate stage larva of *Cymatium nicobaricum*, Western Atlantic, Gulf Stream, same station as (h) above; (m) operculum from larva of *Cymatium nicobaricum*, internal view, specimen from same station as (h) above. Scale = 1 mm in all figures except i, j, and m where it is equivalent to 0.5 mm. Numbers prefixed by "ANSP" are lot numbers in the Academy of Natural Sciences of Philadelphia.

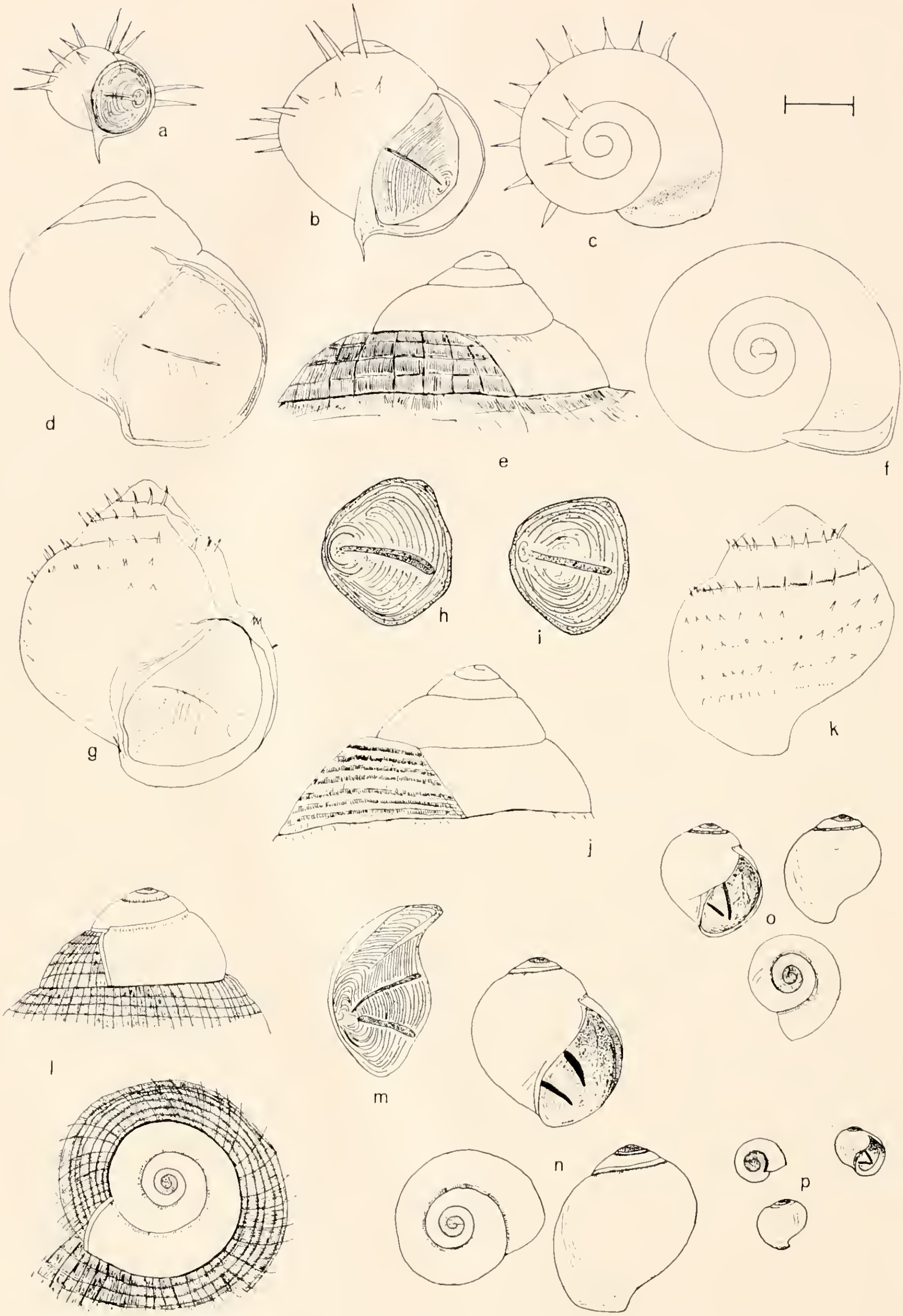
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Figure 2.

hairy shell is dark amber-brown, barely translucent, and uncalcified in the early stages. The protoconch found on museum specimens of this species is smooth and secondarily calcified from within, giving a rather massive appearance (Fig. 1, b). Sometimes there is evidence of periostracal fragments adhering to the protoconch, remains of the elaborate pattern of short, soft spines which give the larvae a hirsute appearance (Fig. 1, a, c, g). The whorls directly following the embryonic shell are cancellate but usually already worn in late larval development (Fig. 1, a). The last three whorls are shouldered. The sutures are normally obscured by the periostracum, which is continuously laid down with the growth of each succeeding whorl. In early larvae the periostracal spines on the first two or three whorls directly following the cancellate whorl are more or less axially arranged (Fig. 1, a). In succeeding whorls the periostracal spines are spirally arranged (Fig. 1, c). With growth of the larva the periostracum of the adapical whorls is overlain with new periostracum upon which the spines are also spirally arranged. On older and larger larval shells the outer parietal lips are glossy and semitransparent in transmitted light but appear very dark brown in reflected light. The operculum of the larva is characteristic, having the nucleus almost central as it also is in the adult (Fig. 1, i; *vide* Clench and Turner, 1957 for illustration of adult operculum). This is probably the species figured but unidentified by Simroth (1895; Pl. VI, Figs. 1–5). Since the protoconch of the eastern Atlantic species *Charonia nodifera* (Lamarck) has not been seen, it is not certain that the larval shell of this species can be separated from *Charonia variegata*. Variations in shell morphology are not consistently related to the geographical distribution of the larvae, and therefore two species of veligers are not distinguishable. The dispersal of *Charonia variegata* larvae by ocean currents is very evident from their widespread geographical distribution throughout the North Atlantic gyre, in the South Equatorial Current, and from scattered records in the Caribbean Sea (Fig. 6).

FIGURE 2. Larval shells, opercula and protoconchs of the Tonnidae and Cassidae: (a) larval shell of the early intermediate stage veliger of *Tonna galea*, Sargasso Sea east of Bermuda, 32°05′N, 59°10′W; (b) intermediate stage larva of *Tonna galea*, North Equatorial Current, 24°34′N, 47°20′W; (c) intermediate stage larva of *Tonna galea*, same specimen as (b) above; (d) shell of fully developed veliger larva of *Tonna galea*, Western Atlantic, Gulf Stream, 36°22′N, 67°53′W; (e) protoconch of *Tonna galea*, apical angle 105°, Western Atlantic, S. Inlet, Lake Worth, Boynton, Florida (ANSP 181564); (f) apical view of fully grown larva of *Tonna galea*, same specimen as (d) above; (g) larval shell of fully grown veliger, *Tonna maculosa*, Western Atlantic, Sargasso Sea, 25°05′N, 57°48′W; (h) larval operculum of intermediate stage veliger of *Tonna maculosa*, viewed from inside, specimen from Gulf Stream between Cape Cod and Bermuda; (i) larval operculum of intermediate stage veliger of *Tonna galea*, viewed from inside, specimen from Gulf Stream between Cape Cod and Bermuda; (j) protoconch of *Tonna maculosa*, apical angle 94°, Western Atlantic, Key West, Florida (Scheltema coll.); (k) larval shell of fully grown veliger, *Tonna maculosa*, same specimen as (g) above; (l) two views of the protoconch of *Phalium granulatum*, Western Atlantic, Matthew Town, Great Inagua, Bahamas (ANSP 173078); (m) operculum from larva of *Phalium granulatum*, viewed from inside, specimen from North Atlantic Drift, 44°11′N, 44°11′W; (n) three views of fully developed larva of *Phalium granulatum*, off the West African coast, 20°32′N, 22°34′W; (o) three views of intermediate stage larva, *Phalium granulatum*, Western Atlantic, Gulf Stream, 38°17′69°36′W; (p) three early stage larvae of *Phalium granulatum*, Western Atlantic, northern end of Gulf Stream, 41°14′N, 57°28′W. Scale = 1 mm in all figures except m, where it is equivalent to 0.5 mm. Numbers prefixed with ANSP refer to museum lot numbers in the collection of the Academy of Natural Sciences of Philadelphia.

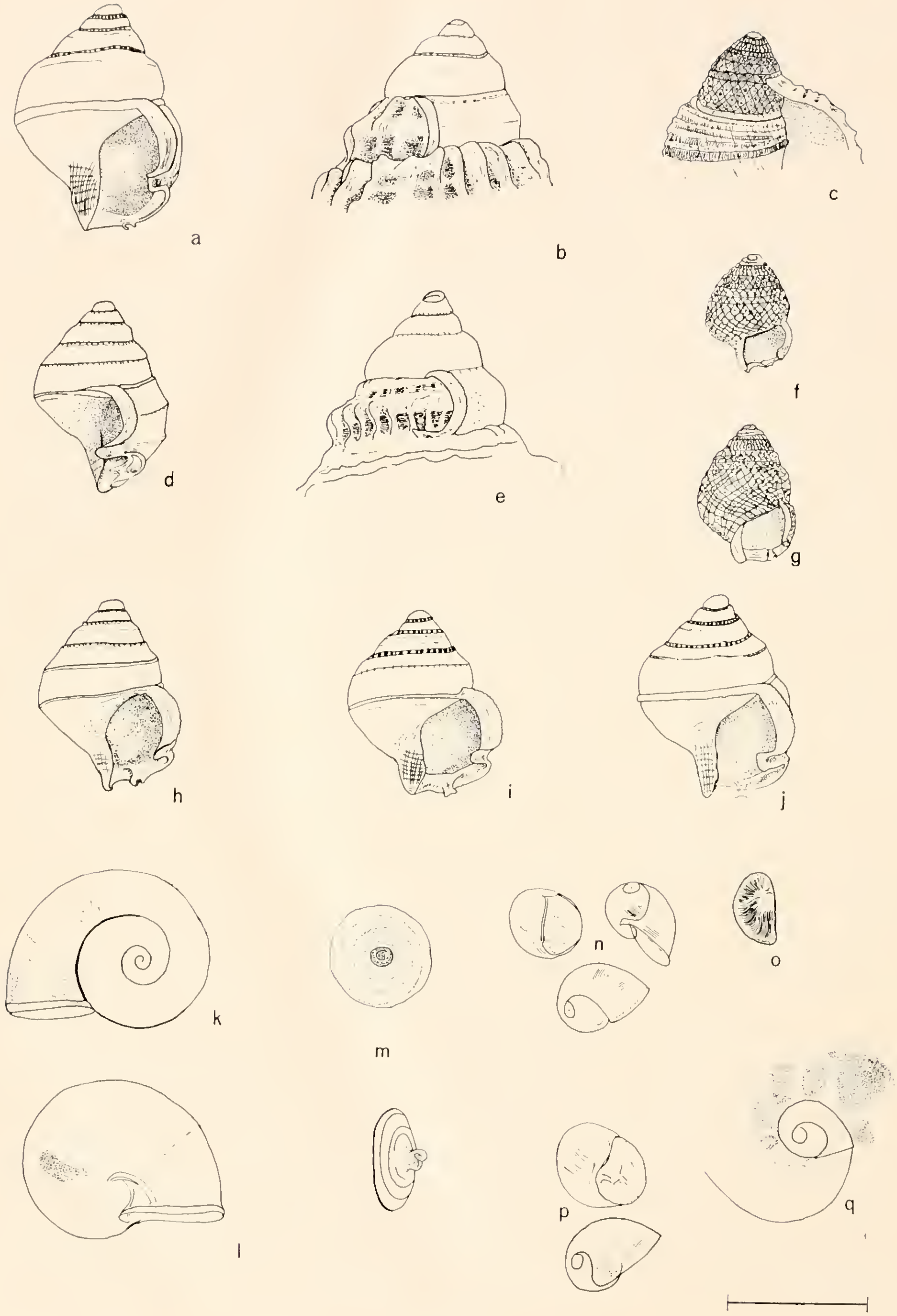
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FIGURE 3.

Family Tonnidae

The Tonnidae or tun shells are large gastropods found in tropical and warm temperate waters throughout the world. Members of this family are restricted to the continental shelf and occur mostly on sandy bottoms. Little is known regarding the life history of most species. Thorson (1940, pages 192–193) has figured the egg mass and early larva of *Tonna* (?) *maculosa* while Knudsen (1950, pages 97–98, Fig. 8) shows an egg mass that he attributes to *Tonna costatum* (Menke). These flat gelatinous masses with "egg spaces" containing about 100 eggs each may have from 350–660 thousand embryos. The eggs are relatively large, 300 microns in diameter, and the early larvae of *Tonna* (?) *maculosa* illustrated by Thorson (1940, page 193, Fig. 15) is about 380 microns in longest dimension. Measurement of the embryonic shell on *Tonna galea* closely corresponds to this, being about 400 microns in length.

The two equatorial Atlantic species are *Tonna galea* (Linné) and *Tonna maculosa* (Dillwyn). Larvae of both these species are encountered in the Gulf Stream.

The early intermediate stage larva of *Tonna galea* has long, straight, fairly stout, spirally arranged, periostracal spines. This stage appears to be similar to that illustrated by Lebour (1945, page 475, Fig. 21) as *Tonna perdix* (Linné). In the later intermediate stage larva, as a new whorl is added, additional spines are also formed (Fig. 2, b, c). The globose shell is smooth, white, and glossy. When completely developed the larva has usually lost all its periostracal spines and then measures about 3.5 mm in length (Fig. 2, d, f). The typical long, drawn-out basal periostracal spine seen in the earlier stages is also frequently lost. At this late stage of development the larva of *Tonna galea* may be confused with that of *Tonna maculosa*. The two differ, however, in the shape of the aperture

FIGURE 3. Larval shells, opercula and protoconchs of some species belonging to the families Muricidae, Ovulidae, Architectonicidae, and Neritidae: (a) *Thais* (?) *rustica* veliger larva, Eastern Atlantic from off West Africa, 22°59′N, 20°30′W; (b) protoconch of *Thais rustica*, Western Atlantic, Port Royal, Jamaica (USNM 442303); (c) protoconch of *Pedicularia sicula decussata*, off coast of Georgia, Western Atlantic, depth 792 m, U. S. Fish. Comm. Sta. No. 2415 (USNM 108408); (d) *Thais haemastoma* larval shell, Western Atlantic east of Bahamas, 25°06′N, 67°42′W; (e) protoconch of *Thais haemastoma*, Western Atlantic, Corpus Christi Bay, Texas (USNM 125561); (f) larval shell of *Pedicularia sicula*, Western Atlantic, Gulf Stream, 36°27′N, 68°01′W; (g) larval shell of *Pedicularia sicula*, Eastern Atlantic, off Azores, 37°14′N, 27°44′W; (h) larval shell of *Thais haemastoma*, Western Atlantic east of Bahamas, same station as (d) above; (i) larval shell of *Thais haemastoma*, Mid-equatorial Atlantic, South Equatorial Current, 02°34′N, 24°03′W; (j) larval shell of *Thais haemastoma*, Eastern Atlantic off West Africa, 08°00′N, 15°15′W; (k) *Philippia krebsii* larval shell, fully developed, Western Atlantic, North Atlantic Drift, 39°26′N, 44°05′W; (l) *Philippia krebsii* larval shell, same as (k) above; (m) operculum of *Philippia krebsii*, inner view and side view showing apophysis; (n) three larval shells of *Smaragdia viridis*, Western Atlantic, Gulf Stream, 30°40′N, 73°40′W; (o) operculum taken from larva of *Smaragdia viridis*, northern end of Gulf Stream, Western Atlantic, 41°26′N, 55°45′W. Arrow shows position of minute reinforcing bar; (p) two larval shells of *Smaragdia viridis*, Eastern Atlantic off West Africa, 07°20′S, 07°23′E; (q) protoconch of *Smaragdia viridis viridemaris* showing larval shell slightly immersed, Western Atlantic, Castle Harbor, Bermuda, depth 7–9 m (ANSP 267608). Scale = 1 mm for all figures. Numbers prefixed by "ANSP" refer to lot numbers in the collection of the Academy of Natural Sciences of Philadelphia. Numbers prefixed by "USNM" are lot numbers from the collection of the United States National Museum, Washington, D. C.

(compare Fig. 2, d with 2, g); the length, number, and angle of the spines (if still present, Fig. 2, k); the apical angle of the spire, usually greater than 100° in *Tonna galea* (Fig. 2, d, e) and less than 100° in *Tonna maculosa* (Fig. 2, g, j); and the shape of the reinforcing bar on the operculum whereby it is attached to the foot musculature (Fig. 2, h, i), being curved in *Tonna maculosa* but straight in *Tonna galea*. Simroth (1895, Pl. IV) under the larval name *Macgillivrayia* has figured *Tonna* larvae. The large operculum (Pl. IV, Fig. 7) is that of *Tonna galea*, but Figs. 1–3 on the same plate are probably *Tonna maculosa*. Larvae of *Tonna galea* were found throughout the North Atlantic gyre, in the South Equatorial Current, off the west coast of Africa, in the mid tropical Atlantic, off the northeast coast of Brazil, and in the Caribbean Sea, whereas those of *Tonna maculosa* were found only in the western Atlantic (Fig. 7).

Family Cassidae

The family Cassidae, known in the vernacular as helmet or bonnet shells, is closely related to the two preceding families (*i.e.*, Tonnidae and Cymatiidae) and like them has a worldwide distribution in tropical and warm temperate waters. The adults live on sandy bottoms or in *Thalassia* beds and feed upon echinoids. The egg mass of *Phalium granulatum* is made up of capsules built into tower-like structures (*vide* Abbott, 1968, Pl. I), and has up to 300 eggs in each capsule. The larvae most frequently encountered in the North Atlantic that belong to the family Cassidae are those of *Phalium granulatum* (Born). This larva is smaller than any of those previously considered here, the shell being somewhat less than 2.5 mm at settlement. The shell is globose (Fig. 2, n, o), though with a somewhat flattened spire, particularly in earlier stages (Fig. 2, p). The sutures are adpressed; the first 1½ whorls are amber-brown while the remainder of the shell is smooth, glossy, and white. The operculum (Fig.2, m) is distinctive with its two strengthening supports.

Two other species of *Phalium* occur in the tropical North Atlantic, (1) *Phalium coronadoi* (Crosse), a rarely collected species known only from the coast of North Carolina and Cuba, and (2) *Phalium saburon* (Bruguière), a species whose distribution overlaps that of *Phalium granulatum*, being found in the Azores, Bay of Biscay, and southward along the west coast of Africa to Ghana. Protoconchs of both these species have been examined and compared to *Phalium granulatum* (Fig. 2, l); *Phalium coronadoi* and *Phalium saburon* both have smaller larvae with wider apical angles.

The geographical distribution of *Phalium granulatum* veliger larvae includes the Gulf Stream, the North Atlantic Drift, the Canary Current off the west coast of Africa, and the South Equatorial Current (Fig. 8).

Family Muricidae

The Muricidae are a family of carnivorous gastropods which feed on bivalves, barnacles, and other gastropods. The genus *Thais* (sometimes relegated to a separate family Thaididae) is represented in the tropical Atlantic by at least four very common species which definitely are known to have pelagic phytoplanktotrophic larvae. The egg capsules of the various species of *Thais* are laid singly rather than in distinctive masses as in some of the Cymatiidae and in *Phalium granulatum* and in most species are usually between 5 and 8 mm in height.

The larval shell of *Thais haemastoma* (Linné) was illustrated by Craven in 1877 as the species *Sinusigera colbeauiana*. Later in 1883 he figured an early post-larval shell which this time he correctly identified as *Thais haemastoma*. Dautzenberg (1889, Pl. II, Fig. 5) has figured an early post-larval shell from the Azores showing details of the protoconch of *Thais haemastoma*. Recently Moore (1961) accurately described the shell of the fully developed larva, but his

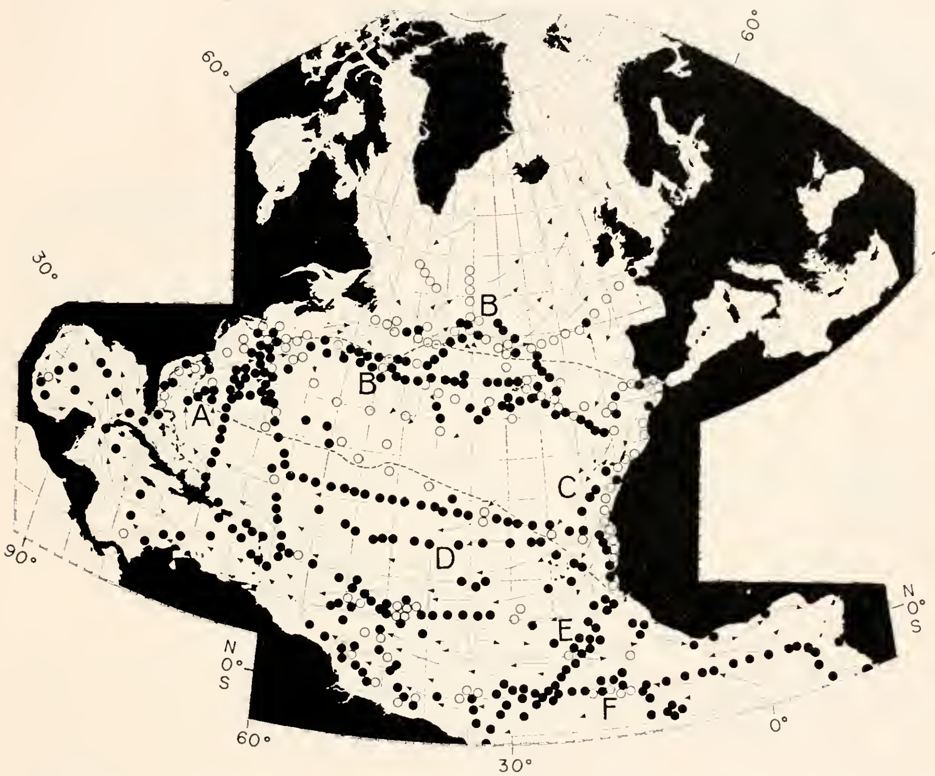

Figure 4. Geographical distribution of plankton tows upon which the present study is based. The darkened circles indicate stations where teleplanic gastropod veligers were found; the open circles denote stations where no such gastropod larvae were taken. In instances when plankton tows were less than one degree apart, points shown on the above and subsequent charts may represent more than one tow. Some stations were repeated at the same location at different times of the year. Locations included in the figure are only those for which entire plankton samples have been examined. Stations appearing on subsequent charts but not on this figure represent positive records from loaned plankton tows. They were not used for negative records because material had been previously sorted from them or preservation was poor, and because they had been collected differently. The dashed lines indicate the 21° C summer and winter isotherms. Major surface currents in the North and tropical Atlantic are designated by arrows and may be identified by reference to letters: A = Gulf Stream, B = North Atlantic Drift, C = Canary Current, D = North Equatorial Current, E = Equatorial Countercurrent, and F = South Equatorial Current.

accompanying illustration (page 26) is confusing and lacks necessary details of shell ornamentation.

The larval shell of *Thais haemastoma* at the completion of growth is about 1.5 mm in length (Fig. 3, d, h, i, j). There are five whorls and an apical embryonic shell. The base of the columella has a cancellate pattern formed from seven or more axial and about an equal number of spiral cords. Below the sutures there are minute axial riblets. The third whorl has a single ribbed spiral cord which continues on the succeeding whorls. The larva is a typical *Sinusigera* with a long projection separating the adapical from the basal portion of the outer lip. A varix, frequently with a serrate adapertural edge, is formed in the adapical portion of the outer lip. The body whorl may have minute punctae extending back about ¼ whorl from the edge of the outer lip. The body whorl is bounded adapically by a spiral band which is continuous with the varix. Certain minute features are lost with preservation and the shell tends to become "chalky" with time.

There are closely related species in the *Thais haemastoma* complex (*e.g.*, *Thais rustica* [Lamarck], *vide* Clench, 1947) which seem to have larvae quite similar to *Thais haemastoma senso stricto* (compare Fig. 3, b and 3, e; the latter protoconch is worn). Certain larvae which were taken off the west coast of Africa and which otherwise appeared identical, differed in having a less calcified shell, being slightly larger, having a somewhat greater apical angle, and having a narrower varix lacking a serrate edge (Fig. 3, a, off west Africa should be compared with Fig. 3, d, h, i, j). These somewhat different west African larvae most closely resemble the protoconch of *Thais rustica*, though this species in the eastern Atlantic has been reported only from St. Helena.

The geographical distribution of *Thais haemastoma* larvae includes the Gulf Stream, the North Altantic Drift, the Canary Current, and the South Equatorial Current (Fig. 9).

Family Architectonicidae

Architectonicidae or sundial shells are a family of subtidal gastropods represented in tropical waters throughout the world. Some subtropical and warm temperate forms are also found. The adults of most known species are restricted to depths of a few meters to about 200 meters near the edge of the continental shelf. Their food is apparently confined to coelenterates, particularly the zoanthid genera *Palythoa* and *Zoanthus* (Robertson, 1967) and also the polyps of scleractinians and actiniarians (Robertson, Scheltema and Adams, 1970). Many Atlantic species are now known to have teleplanic larvae. Moreover, of the ten species found by Marche-Marchad (1969) along the coast of west Africa, seven are also known from the West Indies. The larvae of at least some of these species are eurythermal. *Philippia krebsii* has been found in temperatures from 18.9 to 28.9° C. Other architectonicid larvae have been found at temperatures as low as 13.5° C.

The larval shell of *Philippia krebsii* (Mörch) is illustrated by Robertson (1964, Fig. 4 and Figs. 7–10) and Robertson *et al.* (1970, Figs. 3 and 6). It is hyperstrophic, smooth, glossy, and more or less transparent (Fig. 3, k, l). The operculum is circular, externally concave, with a central internal apophysis that extends into the foot musculature (Fig. 3, m). The apophysis is constricted at the base as is also found in the adults belonging to the subgenus *Psilaxis* (Robertson, 1970a, Fig. 10, page 74). The living larva has four velar lobes with pale orange pigmentation along their borders. The anterior of the foot is darkly pigmented except for a conspicuous white food-rejection tract passing from beneath the mouth to the anterior base of the foot. *Philippia krebsii* is readily distinguished from other species of Architectonicidae in the Atlantic by its large size (1.5 mm along the longest axis) and by the presence of a prominent anal keel (*vide* Robertson, 1964, page 6, Table I). The geographical distribution of *Philippia krebsii* larvae essentially includes all the major surface currents of the tropical and temperate North Atlantic Ocean (Fig. 10).

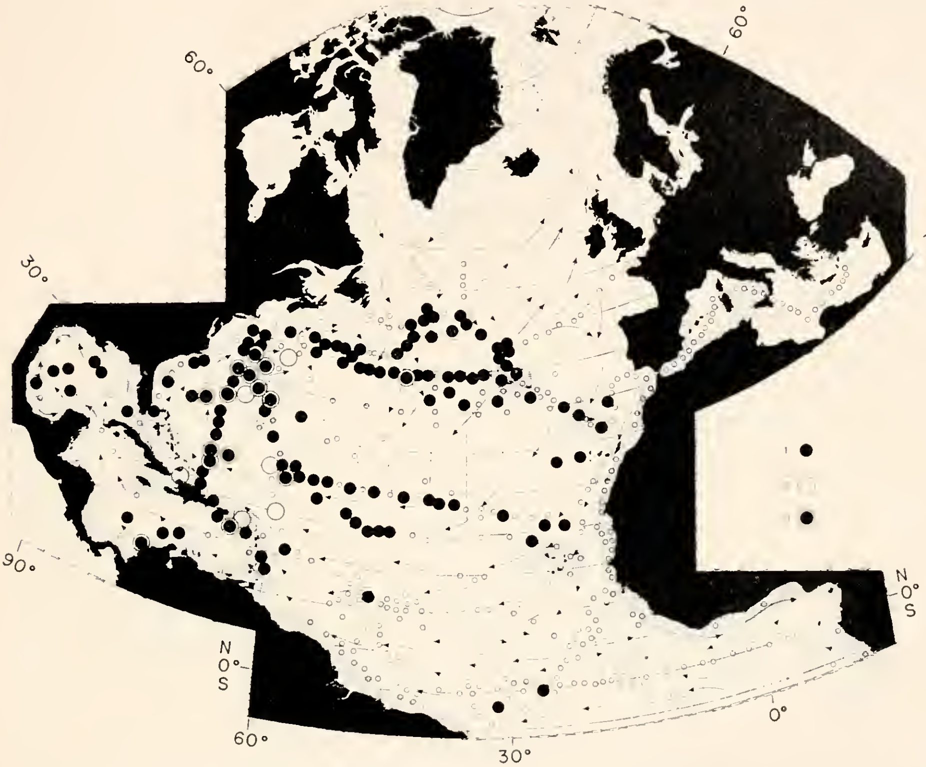

FIGURE 5. Geographical distribution of the larvae of *Cymatium parthenopeum* (von Salis) and *Cymatium nicobaricum* (Röding): 1 = stations with *Cymatium parthenopeum*, 2 = stations with *Cymatium nicobaricum*, 3 = stations where both species were taken. Small open circles are stations where neither species was encountered in tows. Stippled areas show the approximate geographical regions where adult *Cymatium parthenopeum* is to be found to depths of 63 meters (Clench and Turner, 1957). The geographical distribution of post-larval *Cymatium nicobaricum* roughly corresponds to that of *Cymatium parthenopeum* in the western Atlantic, but in the eastern Atlantic *Cymatium nicobaricum* is known only from the Madeira and Canary Islands (Clench and Turner, 1957; Odhner, 1931; Dautzenberg, 1890). Arrows show direction of surface currents.

Family Neritidae

The larval shell of the neritid *Smaragdia viridis* is smooth, glossy white with adpressed sutures (Fig. 3, n, p, q). The embryonic shell is only 120 microns longest dimension, but the fully developed larva is 650 microns along this axis. The living larva has a simple bilobed velum and two small tentacles. The digestive gland has a green, almost chartreuse, cast which is retained for several months after preservation. The small operculum is paucispiral and has a minute reinforcing bar next to the nucleus (Fig. 3, o, see arrow). In later life the adult shell is cryptically colored and is frequently found associated with *Thalassia* in the western Atlantic and *Posidonia* in the Mediterranean. Possibly it may be dispersed on drifting turtle grass (*vide* Menzies, Zaneveld and Pratt, 1967).

The geographical distribution of *Smaragdia viridis* veliger larvae includes the Gulf Stream, the western portion of the North Atlantic Drift, the Canary Current, the South Equatorial Current, and Caribbean Sea (Fig. 11).

Family Ovulidae

The veliger larva of the ovulid *Pedicularia sicula* Swainson is an ovate, amber-brown *Sinusigera*. The embryonic shell, somewhat lighter in color and approximately 125 microns in length, is followed by two spiral whorls with axial costae and a single spiral cord. The two remaining whorls have oblique reticulate ornamentation (Fig. 3, f, g). Sometimes this ornamentation becomes interrupted and confused. The outer lip of the fully developed larval shell has a thickened varix and the typical beak, a projection found on all *Sinusigera*-type veligers. The fully developed larval shell is between 0.9 and 1.0 mm in length.

The protoconch of the post-larva, as is common to members of the Cypraeidae and Ovulidae, becomes completely hidden in the adult by succeeding whorls. It is therefore necessary to have relatively young specimens for comparison with the larva (Fig. 3, c).

A number of names have been applied to the species *Pedicularia sicula* (*vide* Allen, 1956). The limited amount of material examined reveals no good reason for separating the eastern and western Atlantic forms of *Pedicularia* into separate species, and the western Atlantic form, usually known as *Pedicularia decussata* (Gould), should probably be considered no more than a subspecies of the eastern Atlantic and Mediterranean *Pedicularia sicula*. Specimens of North Atlantic species are rare in museum collections so the degree of variability is not well understood.

The protoconch of a Pacific species of *Pedicularia* was first figured by John Dennis MacDonald (1858), assistant-surgeon on H. M. S. Herald in a paper entitled, "On the probable metamorphosis of *Pedicularia* and other forms; affording presumptive evidence that the pelagic Gastropoda, so called, are not adult forms, but, as it were, the larvae of well-known genera. . . ." Dautzenberg (1889) has very well illustrated the protoconch of an Azorean specimen of *Pedicularia sicula* (Pl. IV, Fig. 2a) and the larva described as an unknown ovulid by Lebour (1945, pages 474–475, Fig. 21) off Bermuda is probably *Pedicularia sicula* (*s.l.*). Hedley (1903, Fig. 69, page 342) also figured the protoconch of a *Pedicularia* species from off New South Wales. Other larvae from the family Ovulidae which have been previously described are *Simnia patula* by Lebour (1932), *Simnia spelta* and (?) *Ovula ovum* by Thiriot-Quiévreux (1967). All these forms, though having certain similarities, can be distinguished from *Pedicularia sicula*. Likewise, the protoconchs of Cypraeidae in the Muséum National d'Histoire Naturelle in Paris figured by Ranson (1967) cannot be confused with *Pedicularia* because of differences in the shell ornamentation. However, because of the scarcity of suitable material with protoconchs, it has not been possible to make extensive comparisons with other forms of Cypraeidae and Ovulidae in the tropical North Atlantic.

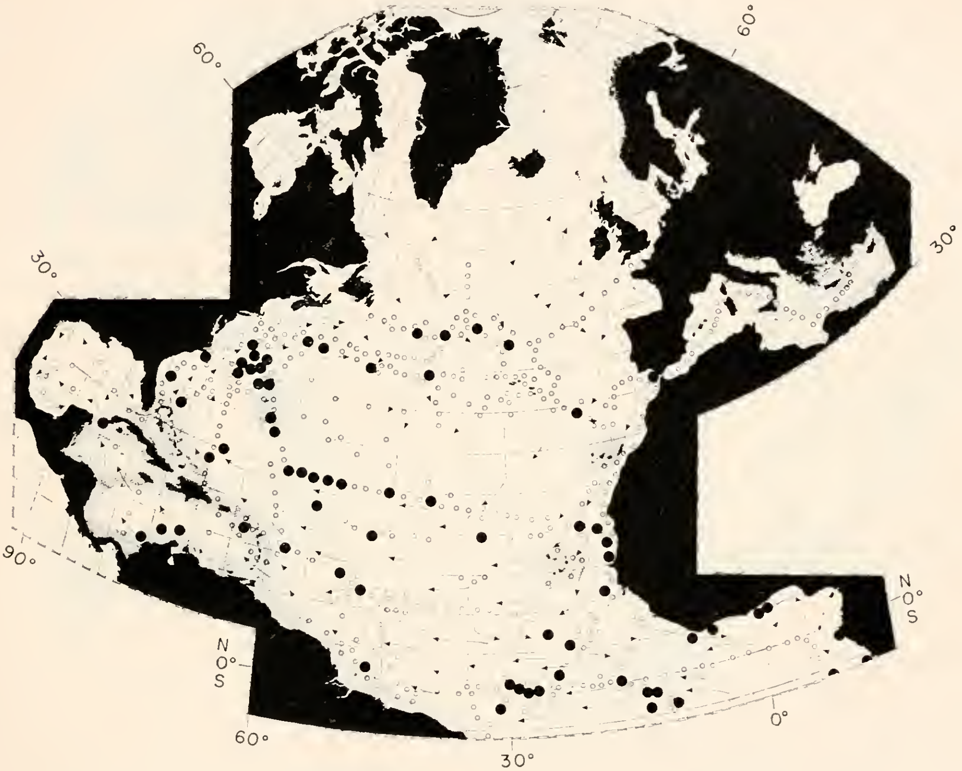

FIGURE 6. Geographical distribution of *Charonia variegata* (Lamarck) larvae: filled black circles = *Charonia variegata* larvae; small open circles = stations where *Charonia variegata* larvae did not occur. The stippling shows approximate geographical regions from which post-larval *Charonia variegata* (*s.s.*) are known (Clench and Turner, 1957) and does not include distributional data of *Charonia nodifera* (Larmarck). Arrows show direction of surface currents.

The larvae of *Pedicularia sicula* (*s.l.*) were found in the Gulf Stream, the North Atlantic Drift, the Mediterranean Sea, and the North and South Equatorial Currents (Fig. 12).

Relationship between the North and Equatorial Atlantic circulation and dispersal of teleplanic gastropod veligers. Two basic patterns of geographical distribution are distinguishable among teleplanic gastropod veligers. The first of these is that found in such forms as *Charonia variegata, Tonna galea, Philippia krebsii, Pedicularia sicula* (*s.l.*), and *Cymatium parthenopeum.* Larvae of these species were found in all three trans-Atlantic surface currents, *viz.,* the North Atlantic Drift and the North and South Equatorial Currents. Some species such as *Phalium granulatum,* *Thais haemastoma,* and *Smaragdia viridis* were missing in the North Equatorial Current but moved in a westerly direction on the South Equatorial Current only.

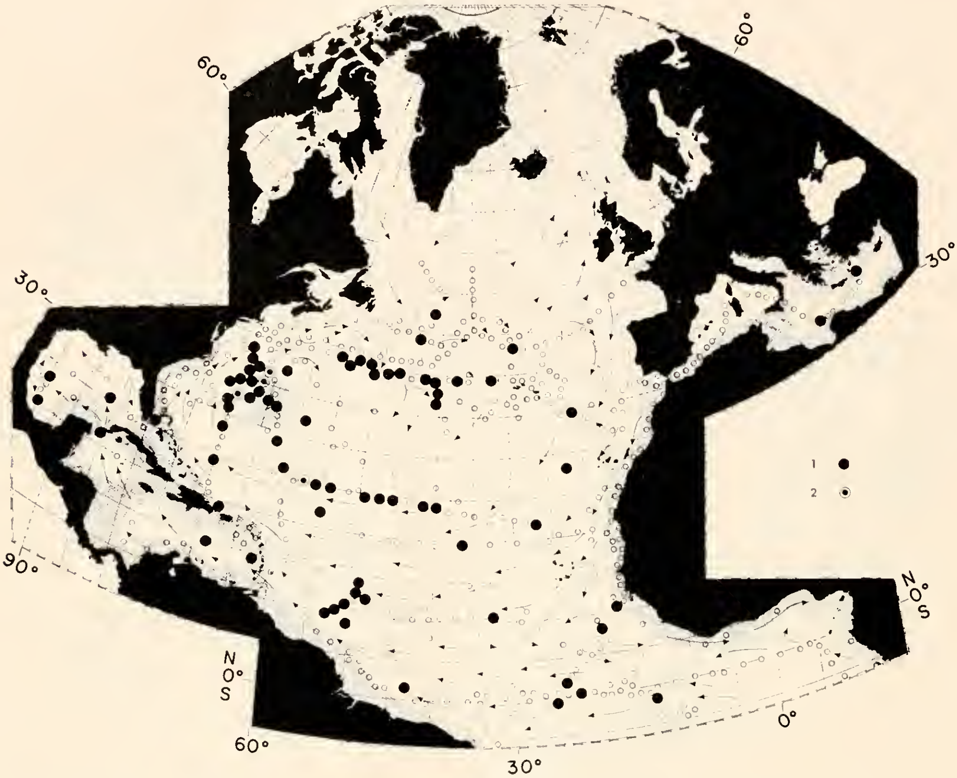

FIGURE 7. Geographical distribution of the larvae of *Tonna galea* (Linné) (=1) and *Tonna maculosa* (Dillwyn) (=2). Small open circles denote stations where no species of *Tonna* veligers were found. Stippled areas show geographical regions where post-larval *Tonna galea* are found to the edge of the continental shelf (Turner, 1946). *Tonna maculosa* post-larvae are known in the eastern Atlantic only from scattered records, specifically in the Canaries (see: Fischer-Piette and Nicklès [1946, pages 45–48] for relevant literature.) Arrows indicate direction of surface currents.

The second basic distributional pattern is that in which larvae were missing in the North Atlantic Drift. Such a distribution is not represented among the ten species considered here, but examples may be found in the gastropod genera *Bursa* and *Cypraea.* These genera evidently have larvae which are dispersed eastwardly on the Equatorial Undercurrent, which provides them with a means for larval transport from the South American to the West African coast (*vide* Scheltema, 1968).

The first distributional pattern is restricted to forms which have eurythermal larvae; otherwise they would not survive the low temperatures of the North Atlantic Drift in which they regularly occur (see 21° C isotherm, Fig. 4). Veligers along the entire periphery of the North Atlantic gyre theoretically can be carried between any two points along its edge. The only limitation to the distance a particular larva may be carried is the length of pelagic life in relation to current velocity.

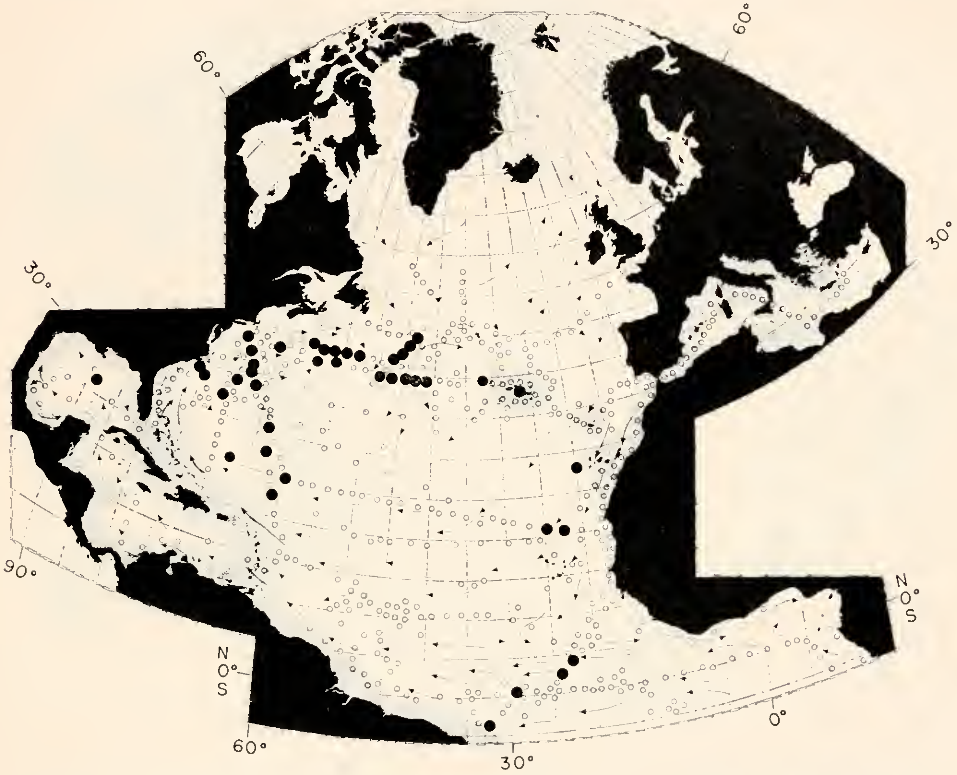

FIGURE 8. Geographical distribution of the veliger larvae of *Phalium granulatum* (Born): filled black circles = larvae of *Phalium granulatum;* small open circles = plankton stations where *Phalium granulatum* was missing from tows. Stippled areas denote geographical regions where *Phalium granulatum* post-larvae have been found. The eastern and western Atlantic forms are designated subspecifically as *Phalium granulatum undulatum* and *Phalium granulatum granulatum,* respectively (Abbott, 1968). Arrows show direction of surface currents.

The second distributional pattern appears to be characteristic of stenothermal tropical forms, excluded from the colder northern route across the Atlantic but effectively carried eastward in the warm Equatorial Undercurrent.

There may of course also be some species in which some factors other than temperature limit the geographical distribution of the larvae.

Duration of pelagic larval life and its relationship to the velocity of trans-Atlantic Ocean currents

Ekman (1953) in his volume *Zoogeography of the Sea* concluded that trans-oceanic drift of larvae is unlikely because "on the whole the planktonic stage is too short to account for dispersal over thousands of kilometers," but he added that "our knowledge of the duration of larval stages in most animals is very meagre" (page 21). Thorson (1961) in a stimulating theoretical paper has summarized data on the length of pelagic larval life of 195 species of bottom invertebrates and concluded that in most instances the period of larval development was too short to account for larval transport across major ocean basins. Among the 18 proso-

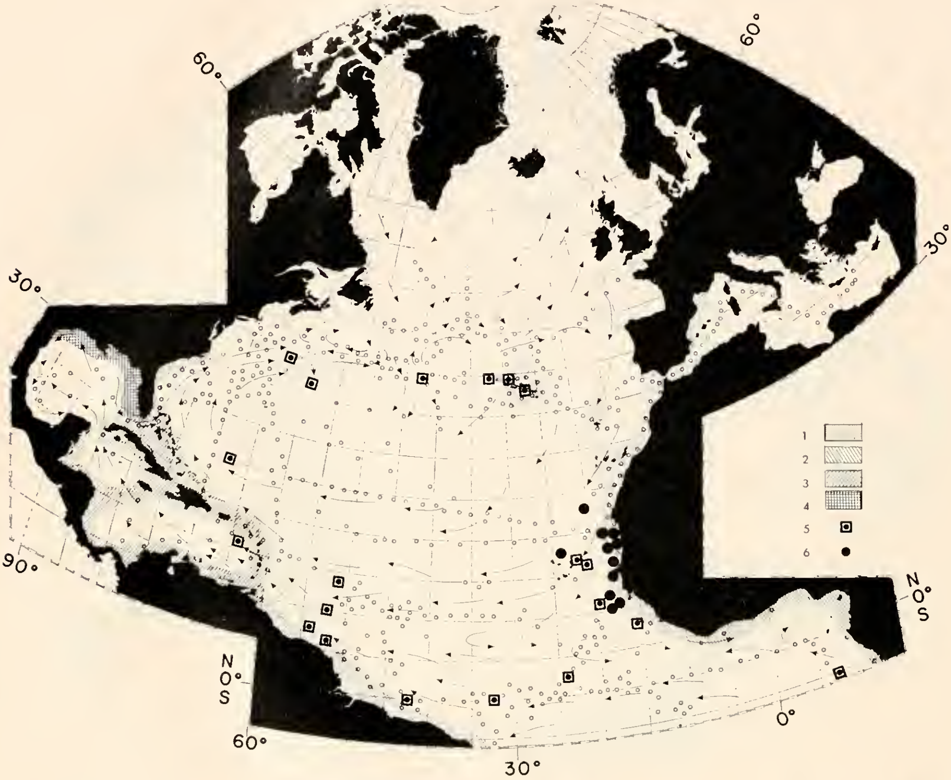

FIGURE 9. Geographical distribution of *Thais haemastoma* larvae. *Thais haemastoma* is known as post-larvae in the eastern Atlantic from two subspecies, *Thais haemastoma haemastoma* (= 1) and *Thais haemastoma forbesi* (= 2); and in the western Atlantic (in addition to the subspecies *Thais haemastoma haemastoma*), from two additional "subspecies," *Thais haemastoma floridana* (= 3) and *Thais haemastoma haysae* (= 4). The adult distribution of these various "subspecies" is taken from Clench (1947) and Knudsen (1956): 5 = larvae of *Thais haemastoma*; 6 = larvae belonging to the *Thais haemastoma* complex but whose specific identity is uncertain (see Figure 3, a). Small open circles are stations where *Thais haemastoma* was not found. Arrow show direction of surface currents.

branch species that were considered, not one had a larva with a development of over 9 weeks. Thorson specifically excluded members of tropical gastropod genera from consideration, because he lacked data for the duration of their pelagic larval life. He acknowledged, however, that "true long-distance larvae . . . seem mainly to be associated with tropical seas and to be rare or lacking in temperate and cold regions" (Thorson, 1961, page 473). Mileikovsky (1966) also believes "that the larval drift of all benthic invertebrates in warmer waters of currents in the tropical regions is more prolonged and lengthy than in their counterparts in waters of temperate and high latitude currents" (page 400, in translation). Since Thorson in his summary article was considering only cold-temperate and boreal species, this information on the length of larval development should not be extrapolated to make conclusions on the dispersal of tropical gastropods in ocean currents.

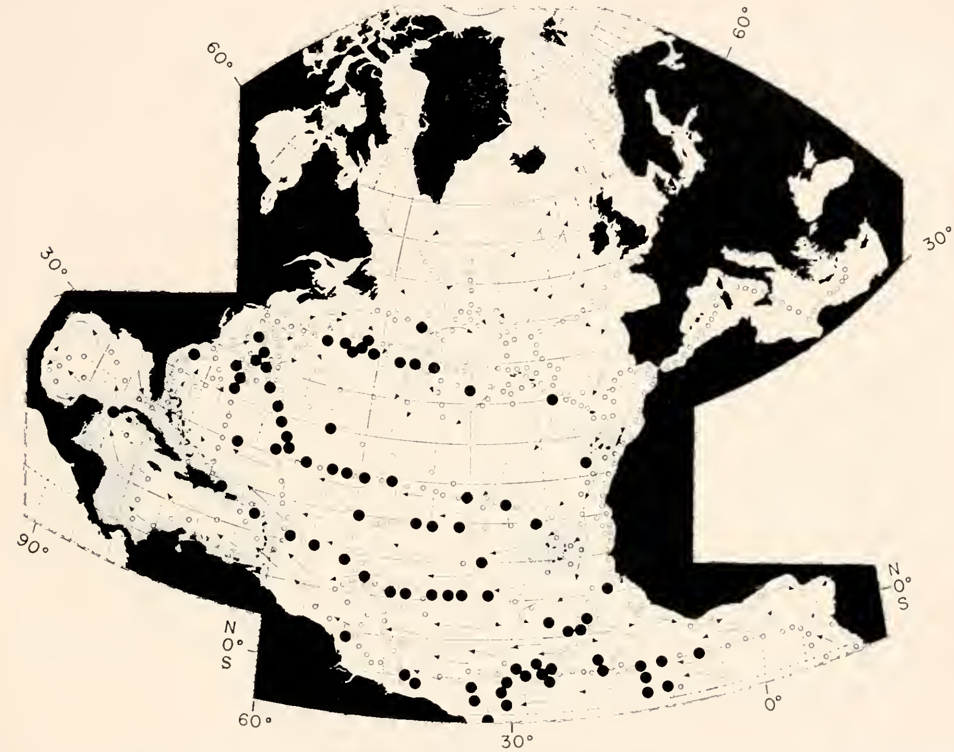

Figure 10. Geographical distribution of *Philippia krebsii* (Mörch) larvae: filled black circles = *Philippia krebsii* larvae; small open circles = plankton stations where *Philippia krebsii* did not occur. The stippling indicates approximate geographical distribution of post-larvae of *Philippia krebsii* (Robertson, 1964; Robertson *et al.*, 1970), which are found to 200 meters depth. The species is presently known in the eastern Atlantic only from the Canary and Cape Verde Islands. Arrows show direction of surface currents.

Lacking experimental data, an approximate measure of the minimum length of pelagic larval development must be obtained indirectly. There are very few data on the growth of any gastropod veliger larvae. However, the growth rate of the prosobranch *Nassarius obsoletus* (Say) is well known under a variety of laboratory conditions (Scheltema, 1967). Let us assume as an approximation that the average shell growth of *Nassarius obsoletus* between 20 and 25° is representative of all phytoplanktotrophic gastropod veliger larvae. If we use this rate and apply it to the measurement of *Cymatium parthenopeum* larvae an estimated value for the minimum time of pelagic larval development may be obtained. Thus in the species *Cymatium parthenopeum* the total pelagic shell growth is represented by the difference in size between the small embryonic shell on top of the cancellate whorl (Fig. 1, j) and that of the fully developed, ready-to-settle larva, almost 2 mm long (Fig. 1, f). This method has been applied to the other species considered (Table I, left-hand column). Admittedly the estimates are only rough, and the accuracy of the method depends partly upon the shell geometry of the species being studied and upon the assumption that early growth is linear. However, in the only case where other data are available, the estimate obtained by this method is in fairly close agreement. Hence, Butler (1954) estimated from field data a minimum pelagic life of about 50 days for *Thais haemastoma,* not far off from my estimate of 62 days.

TABLE I

Estimated duration of the pelagic stage in ten gastropod species having teleplanic larvae

Species	Estimated avg. days required for growth ("developmental period")*	Days held in culture ("delay period")	Total days pelagic
Cymatium nicobaricum	207	113**	320
Cymatium parthenopeum	155	138	293
Charonia variegata	219	57	276
Tonna galea	148	94	242
Tonna maculosa	198	—	(198)
Phalium granulatum	107	—	(107)
Thais haemastoma	62	28**	90
Philippia krebsii	67	*ca.* 7**	74
Smaragdia viridis	25	30	55
Pedicularia sicula	42	—	(42)

* Based on average growth rate of *Nassarius obsoletus* in laboratory culture (*vide* Scheltema, 1967, Table I, page 261).

** These species were successfully held to settlement in the laboratory.

The length of pelagic larval development determined by the method just outlined will most certainly tend to underestimate. Larvae for which the proper algal food is known usually grow faster under ideal laboratory conditions than in their natural environment. Moreover, it is well known that many gastropod species can delay settlement. For example, certain predaceous nudibranchs will delay metamorphosis in the absence of their normal coelenterate prey (Thompson, 1958, 1962; Hadfield and Karlson, 1969), and Fretter and Graham (1962, page 448) report that metamorphosis of *Odostomia* (Pyramidellidae) is accelerated by the presence of the polychaete worm *Pomatoceros* and delayed by its absence. Other gastropod larvae have a more general response. Thus the intertidal snail *Nassarius obsoletus,* primarily a deposit-feeder, delays settlement in the absence of an appropriate sediment (Scheltema, 1956, 1961), and Kiseleva (1966, 1967) has shown that the prosobranchs *Rissoa splendida* and *Bittium reticulatum* settle preferentially on the alga *Cystoseira.* In some species settlement may be simply a tactile response to a surface, apparently the case with the architectonicid *Philippia krebsii.*

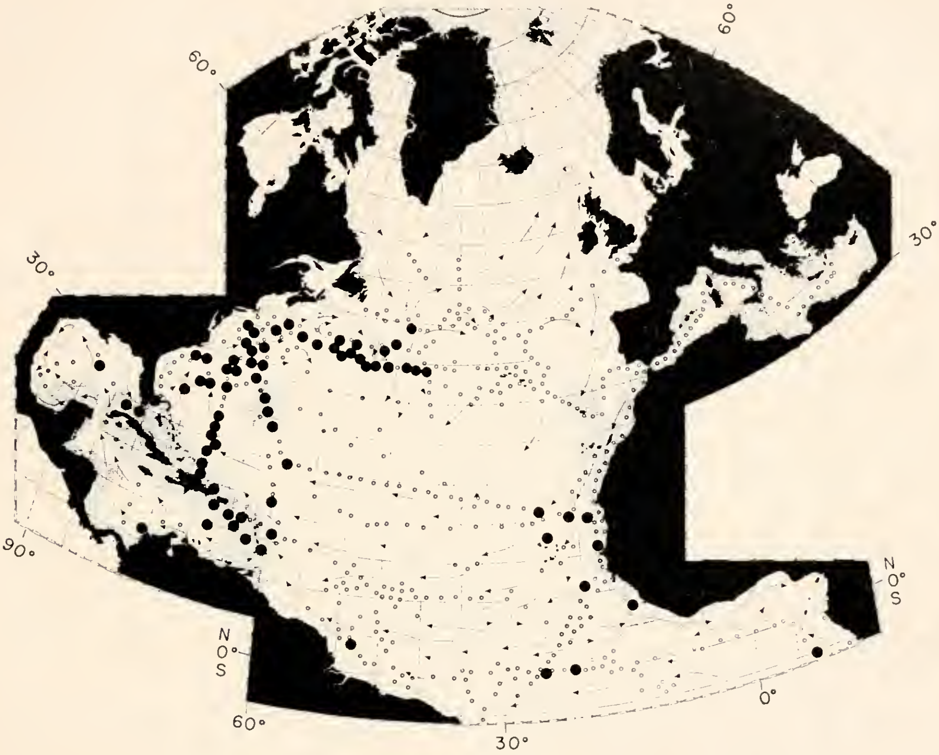

FIGURE 11. Geographical distribution of the larvae of *Smaragdia viridis* (Linné): filled black circles = larvae of *Smaragdia viridis;* small open circles = plankton stations where *Smaragdia viridis* was not found. Stippled areas show approximate regions where post-larval *Smaragdia viridis* is found; eastern and western Atlantic subspecies are respectively designated *Smaragdia viridis viridis* (Linné) and *Smaragdia viridis viridemaris* (Maury). Distributional data are compiled from the collections of the Museum of Comparative Zoology and the Philadelphia Academy of Natural Sciences. Arrows show direction of surface currents.

The larval development of many phytoplanktotrophic gastropod veligers can be divided for convenience into two periods (Scheltema, 1967). The first of these—one of rapid growth and morphological development—may be termed the "developmental period." Its end is marked by the inflection point on the cumulative growth curve and the completion of external morphological development (Scheltema, 1967, page 260, Fig. 4). Larvae are ready to settle at this time. Estimates of the duration of pelagic larval development based on the differences in size between the embryonic and fully developed larval shell are roughly equivalent to the length of the developmental period.

The second period, termed the "delay period," is terminated by a settlement response. In species with very specific post-larval requirements, such a response can be very precise. Very little further growth occurs during the delay period. Many veliger larvae captured in the open sea have completed the developmental period and reached the delay period. Indeed, it was the constancy in size that confused Craven (1883) into believing that the veliger larvae of the *Sinusigera*-type (Fig. 3, a–j) were fully developed holoplanktonic species. He writes, "The two principal arguments in favor of this view were the great distances from land at which they were often found and the constant dimensions of each species" (page 141). It is the delay period which gives to many species the greatest flexibility in the length of their pelagic larval life.

TABLE II

Velocity of the eastwardly and westwardly flowing currents of the north and tropical Atlantic Ocean

Region	Estimated current velocity km/hr	Estimated number of days required for trans-Atlantic drift
North Atlantic Drift (Bahamas to Azores)	0.5–1.3	128–300
(Bahamas to N. W. Africa)	0.9	400
North Equatorial Current		
(N. W. Africa to West Indies)	0.9–1.2	128–171
South Equatorial Current		
(Gulf of Guinea to Brazil)	1.0–3.2	60–154
Equatorial Undercurrent		
(Brazil to Gulf of Guinea)	2.0	96

Data from (1) Pilot Charts, U. S. Hydrographic Office; (2) direct measurements: Dickson and Evans, 1956, North Equatorial Current; Stalcup and Metcalf, 1966, Equatorial Undercurrent; and (3) drift bottle data: Guppy, 1917; Bumpus and Lauzier, 1965; and W.H.O.I. files courtesy of Mr. Dean F. Bumpus.

I have attempted to obtain some measure of the potential length of the delay period experimentally by culturing larvae caught at sea in plankton nets. The endurance of some veliger species under laboratory conditions is truly remarkable (Table I, column designated "delay period"). Thus *Cymatium nicobaricum* was held for 113 days and *Cymatium parthenopeum* for 138 days. Not all species were successfully held in culture, and some species were not caught during the time that culture observations were made.

Addition of the estimated time of the developmental period to that of the delay period probably gives a better estimate of the maximum pelagic life of a species of gastropod than the estimated developmental period alone (last column, Table I).

One may compare these data for the duration of pelagic life with the current velocities of the major eastwardly and westwardly flowing currents of the North Atlantic Ocean (Table II). Under favorable hydrographic conditions the duration of pelagic larval life of *Cymatium nicobaricum*, *Cymatium parthenopeum*, *Charonia variegata*, *Tonna galea*, *Tonna maculosa*, and probably *Phalium granulatum* are all sufficiently long to account for their trans-Atlantic dispersal over any of the four major routes.

The remaining four species, *Thais haemastoma*, *Philippia krebsii*, *Smaragdia viridis*, and *Pedicularia sicula* seem to have but little opportunity for trans-Atlantic transport either by way of the North Atlantic Drift or the North Equatorial Current, and only under the most favorable conditions could cross in the South Equatorial Current and the Equatorial Undercurrent. Yet three of the four remaining species under consideration have larvae throughout the entire North Atlantic Drift and two are found also along the North Equatorial Current (see Figs. 9, 10, 11, and 12).

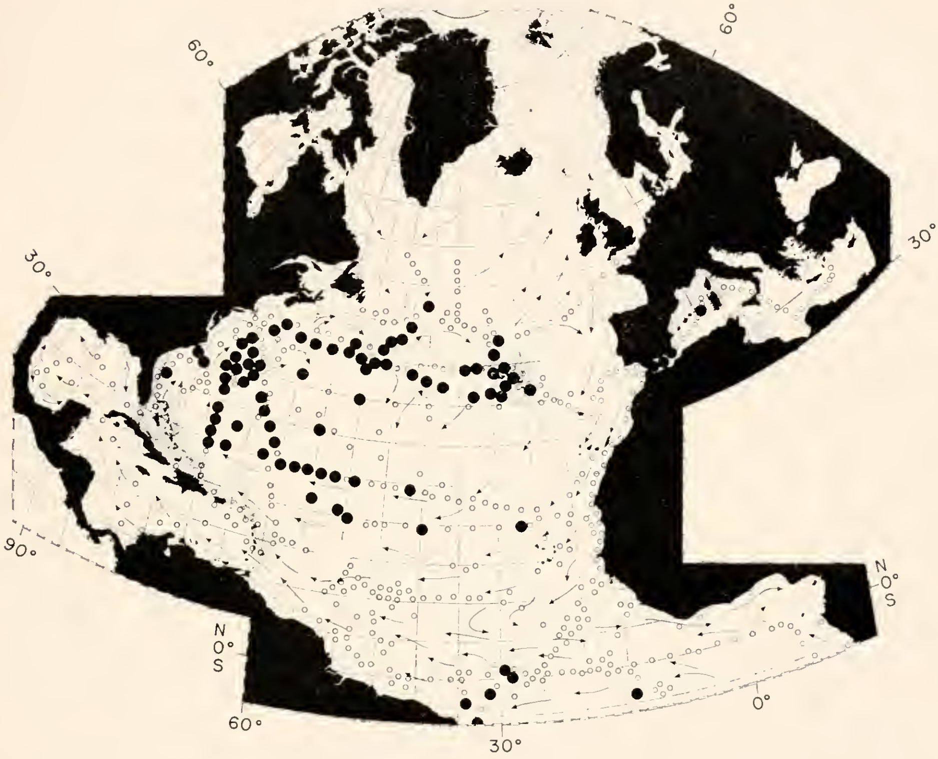

FIGURE 12. Geographical distribution of the larvae of *Pedicularia sicula* Swainson: filled black circles = larvae of *Pedicularia sicula*; small open circles = stations at which larvae of *Pedicularia sicula* were not taken. Stippled areas designate geographical regions from which the post-larvae are known (Allen, 1956). The western Atlantic form *Pedicularia decussata* Gould should probably be regarded as no more than a subspecies of *Pedicularia sicula* (*s.s.*). Arrows show direction of surface currents.

The answer to this paradox must be that the duration of pelagic larval development among these species is longer than estimated. Three possibilities suggest themselves. The first is that the developmental period is much extended under conditions of the open sea. It is known, for example, from observations in the laboratory that when food is less than optimal, length of the developmental period can be very greatly extended, in some species evidently for months (Millar and Scott, 1967; Turner and Johnson, 1968; personal unpublished observations). It is of course also well known that the amount of phytoplankton in the open sea is very substantially less than in neritic waters. Laboratory experiments show that a reduction in water temperature may extend the length of the developmental period of gastropod veligers up to fifty per cent (Scheltema, 1967, in *Nassarius obsoletus*). This observation is particularly relevant to the duration of pelagic larval development in the North Atlantic Drift as during some months of the year the surface-water temperature there can be ten degrees lower than in the tropical Atlantic.

The second possibility is that the potential length of the delay period may extend over a longer time than has been estimated. Species which as adults have very particular habitats or which prey on specific organisms as food may be suspected of having marked settlement responses. Thus *Pedicularia sicula*, as an adult found only on stylasterine hydrocorals (Robertson, 1970b), may be expected to have a highly developed settlement response. A delay in settlement until a suitable environment is encountered can greatly increase the length of the delay period and the total duration of pelagic existence.

The third possibility is that certain species having relatively small egg capsules may attach these to floating objects, and that the larvae found in the open sea are released subsequently from these capsules. This is clearly not a possibility for the first six species in Table I as their egg masses are quite large and are never attached to floating objects. The capsules of *Thais haemastoma* are relatively small and conceivably could be attached to drifting materials. It is possible that occasionally the adults of certain small species such as *Smaragdia viridis* are mechanically transported, in this instance on turtle grass with which *Smaragdia viridis* is normally associated. Clench (1947, page 76) cites the case of a specimen of *Thais haemastoma* taken from a floating log 300 miles northwest of the Madeira Islands. However, floating objects are seldom seen in the open sea, even in sea-lanes, and in any case could not possibly account for the large numbers of larvae found there.

It is now possible to summarize more specifically the case of the four species in question.

Thais haemastoma is the only species on the list in Table I which shows marked seasonality in its reproduction. Its larva has been found in the North Atlantic Drift only in the autumn, when the falling sea-surface temperature may extend the duration of its larval development (sea-surface temperature is about 21° C during October).

Philippia krebsii is probably able to extend its larval development beyond that suggested by the data in Table I (see also Robertson, 1964, for estimate of length of larval development). In the absence of a tactile stimulus, it can probably greatly extend the duration of its pelagic existence (Robertson *et al.*, 1970).

Probably the larvae of *Smaragdia viridis* are infrequently dispersed across the North Atlantic Drift because of their relatively short planktonic development. The alternative of the adults' drifting on turtle grass is, however, quite possible.

Finally, the delay period of *Pedicularia sicula* larvae, for which there are no data, probably can be extended over long periods. This seems the most reasonable hypothesis to explain its widespread occurrence in all the North Atlantic surface currents (Fig. 12).

Probability and frequency of long-distance dispersal of veliger larvae

Estimates of the probability and frequency of dispersal from drift-bottle data and survival rates

Larval transport between continents has often been likened to a sweepstakes, because it is believed that the odds that any specific larva will drift across the Atlantic Basin are very small. It is generally concluded from such a quasi-quantitative statement that only rarely can a larva be dispersed in either direction across the Atlantic.

The probability of successful trans-Atlantic larval dispersal, p, depends upon the drift coefficient, p_d, and the survival coefficient, p_s, such that:

$$p = p_d p_s \tag{1}$$

TABLE III

*Percentage drift-bottles released along the North American Coast and subsequently recovered in the Eastern Atlantic Ocean (based on 156,276 bottles)**

Region of recovery	Percentage of total bottles released
Northern Europe (Great Britain, Ireland, Denmark, Norway)	0.10
Azores	0.05
Southern Europe (France, Spain, Portugal)	0.04
Northwest Africa (Including Canary Islands)	0.01
Total percentage recovery in Eastern Atlantic	0.20

* Computed from data in files of Woods Hole Oceanographic Institution and made available through the courtesy of Mr. Dean F. Bumpus.

Specifically, the drift coefficient can be defined as the likelihood that larvae will enter and remain within a current that will transport them across the Atlantic Ocean Basin rather than back onto their own shore. The value of p_d depends upon (1) the distance between the parent population and the ultimate destination of the larvae, that is, the greater the distance the less likelihood for success, and (2) the size of the target area, or, the amount of coastline which the larvae are likely to encounter. An approximate value for the drift coefficient may be derived from a knowledge of drift-bottle and drift-card recoveries. It is assumed that if a larva remains within the surface currents, it will be carried in the same manner as the drift bottles or cards and that consequently the likelihood of its transport across the Atlantic Ocean, other factors being equal, will be similar to that of such bottles or cards. The per cent recovery in both directions, west to east along the North Atlantic Drift and east to west along the North Equatorial Current, is remarkably similar (Tables III and IV), even though the data are from entirely different sources and the sample sizes differ by two orders of magnitude. From these recovery data an average value of 1.9×10^{-3} may be assigned to p_d. As probably not all bottles that actually make the crossing are eventually recovered, the value of p_d is doubtlessly conservative.

The survival coefficient p_s is the probability that a pelagic phytoplanktotropic larva will survive to settlement without regard to where it may be dispersed. It must include possibility of mortality by predation, disease, or starvation. Because such information is not known for any tropical gastropod veliger, the value for larval survival, p_s, must be derived by an indirect method.

Over the long course of time it is evident that each member of a species must reproduce itself at least once during its lifetime; otherwise the species will decrease in numbers to eventual extinction. Therefore the minimum survival of any viable species must on the average be such that for each member there is produced during its lifetime one larva that ultimately survives to sexual maturity. Thus in a dioecious species:

TABLE IV

*Percentage drift-cards released along the North-West African Coast and subsequently recovered in the Western Atlantic Ocean (based on 1,800 cards)**

Region of recovery	Percentage of total
Anguilla (British West Indies)	0.06
East Coast of Florida (Cocoa Beach)	0.06
Outer Banks (North Carolina)	0.06
Total percentage recovery in Western Atlantic	0.18

* Data computed from Stander, Shannon and Campbell, 1969, Fig. 1, page 296; Table I, page 294–295.

$$p_s = \frac{2}{t_f} \tag{2}$$

where t_f is the fecundity or total number of eggs produced per female.

The numerator "2" in the expression on the right-hand side of the equation assumes an equal number of males and females in the population. The equation further assumes that chances for survival are equally good in coastal and off-shore waters. The expression probably tends somewhat to underestimate larval survival because it includes post-larval mortality. However, the latter is regarded as being very small compared to the mortality of larvae.

Selected examples can now be considered. Members of the genus *Tonna* are known to produce broad, flat, ribbon-like egg masses with *ca.* 300,000 to 600,000 eggs. Thorson (1940, page 192) estimated that *Tonna* (?) *maculosa* in the Iranian Gulf produced 350,000 eggs and Knudsen (1950, page 98) describes an egg mass of a *Tonna* from off West Africa with 660,000 eggs. It is not known how many egg masses a female produces, but for ease in calculation let us assume that each spawns five per year over a two-year period and every mass contains

500,000 eggs. The probability that a particular larva from an egg mass will survive and ultimately reach the opposite coast of the Atlantic Basin will then be:

$$p = p_d \cdot \frac{2}{t_f} = [1.9 \times 10^{-3}][4 \times 10^{-7}] = 7.6 \times 10^{-10} \tag{3}$$

or approximately one in eighty billion. Butler (1954) reports that each female *Thais haemastoma* produces 500,000 eggs per season, and a result similar to that of *Tonna* will then be obtained. Thus the probability that any specific larva will be transported across the Atlantic Basin is very small. However, what concerns us here is not, as in a sweepstakes, the likelihood that a particular horse will finish, but rather the total number that will complete the race.

It does not only matter what the odds for dispersal of a particular larva may be; what is also important is the total number of larvae that are dispersed (*vide* Simpson, 1952, pages 167–168). If enough larvae are produced, the chances may become very good that at least some veligers will successfully drift across the Atlantic.

Therefore the frequency of trans-Atlantic dispersal is dependent, not only on the probability of such an event, but also on the number of larvae produced by the parent population. Thus,

$$d = pt \tag{4}$$

where d is the frequency of dispersal, and t is the number of larvae produced by a population. The value of t is a function of the population size, t_p, and the number of eggs produced per female, t_f. In a dioecious species, assuming that half of the population is female, the total number of larvae produced is $t_p/2 \cdot t_f$. Assuming once more that the survival coefficient is $2/t_f$ and substituting in (4) above,

$$d = \left[p_d \cdot \frac{2}{t_f}\right] \cdot \left[\frac{t_p}{2} \cdot t_f\right] = p_d t_p \tag{5}$$

which is simply to write that the frequency of dispersal depends upon (1) the probability that larvae will be carried across the Atlantic by the major ocean currents and (2) the size of the parent population from which the larvae originate. Since p_d, the drift coefficient can be assumed to be essentially constant, the frequency of long distance dispersal on the average may be considered as proportional to the size of the population from which the larvae originate (Fig. 10).

But is is characteristic of benthic marine species that have pelagic larvae, that their adult populations on the bottom vary greatly from one year to the next (Thorson, 1946, page 436, Fig. 199; Coe, 1956). Such large changes in population size most frequently reflect the success or failure of planktonic larval development. If large variations in larval survival occur from one year to the next, it follows that the frequency of long-distance dispersal must also fluctuate widely. There are, however, surprisingly few continuous records over long periods which measure such fluctuations. Among the best of such published data are those collected by Loosanoff (1966, 1964) on the settlement of the American oyster *Crassostrea virginica* and its principal predator, the seastar *Asterias forbesi*, in Long Island Sound over a period of 25 years. Between 1937 and 1961, Loosanoff found extremes in the intensity of settlement from 3 to 42,623 organisms per hundred shell faces ($\cong 0.25$ M²). The median intensity of settlement was between one thousand and ten thousand larvae per unit area; during sixteen of the twenty-five years the settlement intensity fell within this range (Fig. 14).

In *Asterias forbesi* the intensity of settlement was more variable from year to year. Thus, during six of the twenty-five years of observation, the density of settlement per hundred shell faces was between 1 and 10 organisms; during six other years it fell between 10 and 100 organisms; for nine years it was between 100 and 1000 organisms, and finally during two years it fell between 1000 and 10,000 organisms (Fig. 14). From these and other available data (Thorson, 1946; Coe, 1956) it appears that in most instances the fluctuation in successful settlement will lie within four and certainly five orders of magnitude. These data allow one to predict that the variation in the frequency of long-distance dispersal will probably also fall within similar limits (Fig. 13).

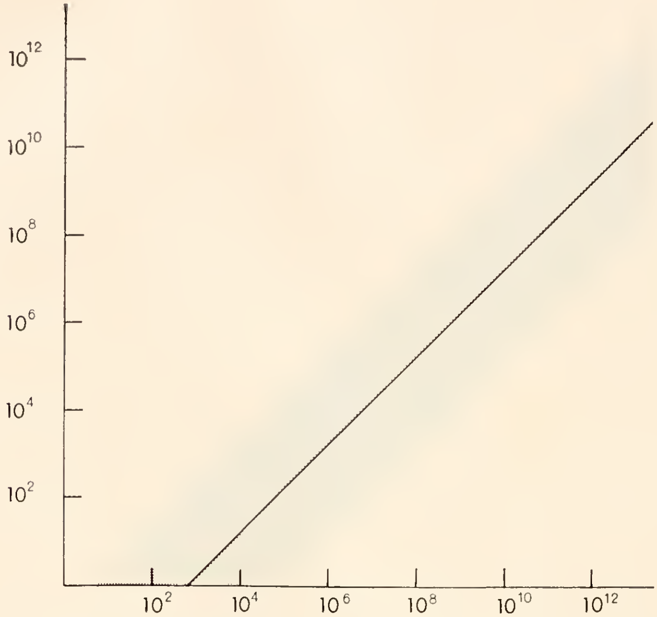

FIGURE 13. Relationship between frequency of larval dispersal (d) and population size (t_p). The horizontal axis shows population size, and the vertical axis frequency of dispersal. In this example a value of 1.9×10^{-3} is assumed for the drift coefficient (p_d). This value is approximate for p_d in the North and Equatorial Atlantic Ocean. The curve shows the expected frequency of larval dispersal (d) computed from various assumed population sizes when only one larva survives for each adult member of the parent population. Fluctuations in larval survival for most benthic species are expected to lie within the stippled region (*vide* Fig. 11; Thorson, 1946, page 439, Fig. 199; Coe, 1956; Loosanoff, 1964, 1966).

In considering the frequency of long-distance dispersal it has been necessary to assume that the duration of larval development is sufficient to allow transoceanic dispersal, because it is not possible to arrive at a coefficient which uniquely represents the length of larval life and yet is biologically unrelated to some character defined by the survival coefficient, p_s.

The theoretical model shows that, over long periods of time (decades or more), the frequency of dispersal is largely dependent upon the parent population size, but that over shorter periods it may be more closely related to survival of the larvae.

Estimates of dispersal frequency from occurrence of larvae in the plankton

A further consideration of evidence from the plankton tows and its relationship to the preceding theoretical model can now be made. It is first necessary to determine the limitations of the collection method. If a plankton net of three-quarter-meter diameter is towed at approximately 5.5 km/hr (3 knots) for twenty minutes and the efficiency of the net is assumed to be ninety per cent, then the capture of a single organism represents 1.35×10^{-3} organisms per cubic meter. This figure represents the lower limits of sensitivity obtained by the method of capture. When larvae occur at even lower concentrations, they will not be captured in every plankton tow taken. When their concentration is greater than $1.35 \times 10^{-3}/m^3$ they will be expected to occur more than once in each sample. The numbers of larvae actually taken varied with the species and the distance from the point of their origin. In my samples the numbers ranged from one to over 500 per tow; however, it seldom occurred that more than ten gastropod veliger larvae of any particular species were taken in a mid-ocean sample. Thus the concentration of larvae in most tows, by comparison with the more common holoplanktonic species, was very low.

Table V

Number of gastropod veligers present in each of the major east-west currents of the North Atlantic Ocean when homogeneous distribution and minimal detectable concentrations of larvae by the present method of sampling are assumed

(Minimum detectable concentration = 1.35×10^{-3} larvae/m^3)

	Volume of water* (m^3)	Total number larvae at conc. of one/tow
North Atlantic Drift	2.45×10^5	3.31×10^{11}
North Equatorial Current	3.43×10^5	4.63×10^{11}
South Equatorial Current	2.26×10^5	3.05×10^{11}
Equatorial Undercurrent	0.98×10^5	1.32×10^{11}

* In computing the sea-surface area of each major ocean current the following co-ordinates when connected by straight lines were arbitrarily considered to enclose the currents: North Atlantic Drift—45°N–60°W; 45°N–20°W; 35°N–10°W; 35°N–72°W; North Equatorial Current—25°N–65°W; 25°N–20°W; 10°N–25°W; 10°N–55°W; South Equatorial Current—5°N–50°W; 5°N–20°W; 5°S–7°E; 5°S–35°W; Equatorial Undercurrent—2°N–35°W; 2°N–5°E; 2°S–5°E; 2°S–35°W.

Even though the concentration of larvae per cubic meter may be small, the volume of water in which the veligers are found is very large. If one arbitrarily delimits the surface area of each of the major east-west currents of the North and Equatorial Atlantic and considers only the upper 50 m (or in the case of the

Equatorial Undercurrent a vertical distance between 50 and 100 m depth) one can compute the volume of water in which most of the larvae are to be found (Table V). Assuming the lowest measurable concentration of one larva per tow, and considering the case in which a single veliger occurs in each sample of a trans-oceanic section, one can now roughly calculate the number of larvae that ought to be present throughout the length of any such current at one instant in time. Thus if the concentration of gastropod veligers is $1.35 \times 10^{-3}/\text{m}^3$, the number of larvae in the North Equatorial Current will be

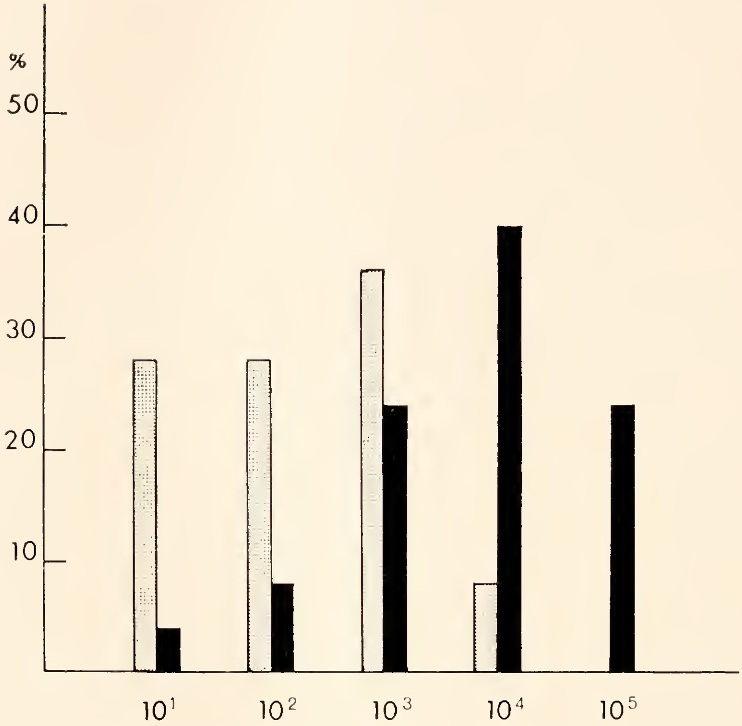

FIGURE 14. Intensity of larval settlement of the sea-star *Asteria forbesi* (stippled bars) and the oyster *Crassostrea virginica* (solid bars) over a period of twenty-five years. The ordinate gives the percentage of years during which settlement occurred at a density value (number/0.25 m^2) shown on the abscissa. The success of settlement is regarded as being directly related to larval survival and, at least in the species represented here, the median density of settlement is *approximately* equal to the survival when $p_s = 2/t_f$ (*i.e.*, when each individual in the population gives rise to one offspring). There are other species where the latter relationship is probably not true, specifically, when the frequency-density curve is skewed (data taken from Loosanoff, 1964, 1966).

$$[3.43 \times 10^5 \text{ km}^3] \cdot [1.35 \times 10^6 \text{ larvae/km}^3] = 4.63 \times 10^{11}$$

or about 460 billion veligers. East-west sections made across the Atlantic in the North Equatorial Current during the months of January and June show that, in half of the species considered, larvae occurred during both the winter and summer months. The implication is that the total larvae present in the current may approximately assume a steady state and that as larvae are removed from the western extremity of the current, new ones are added at the eastern end. Assuming a travel time for the larvae of about 170 days (Table II), the number of larvae transported across the Atlantic Basin on the North Equatorial Current each year will be somewhat over two times the total number of larvae present at any one instant.

Perhaps these computations are somewhat fanciful. Actually the larvae are not known to be uniformly distributed, although uniform distribution is of course the underlying assumption in the foregoing calculations. Indeed, none of the species are found in every sample, whereas, on the other hand, many frequently occurred in numbers greater than one per tow. What the computations do clearly indicate is that large numbers of larvae may be dispersed and yet be completely undetected by the present method of sampling. If the larvae of a species can be found in mid-ocean with consistent regularity, it is likely that it frequently will be dispersed across the Atlantic.

TABLE VI

Relative frequency at which ten species of gastropod veliger larvae occur in plankton samples from the warm-temperate and tropical surface waters of North Atlantic Ocean (based on 658 samples)

Species	Number of stations found	Percentage of stations found
Cymatium parthenopeum	177	26.9
Philippia krebsii	136	20.7
Pedicularia sicula	114	17.3
Smaragdia viridis	95	14.4
Charonia variegata	91	13.8
Tonna galea	89	13.5
Phalium granulatum	33	5.0
Thais haemastoma	32	4.9
Cymatium nicobaricum	28	4.3
Tonna maculosa	4	0.6

The fate of the larvae after they reach their destination is yet another matter. The number of larvae required to have any genetic impact on the recipient population must depend upon the selective advantage that the characters carried by the larvae from their parent population may have in the new environment.

Relative rates of genetic exchange and the apparent effect on geographically separated populations of marine gastropods

The frequency with which a species of larva is found in a series of tows along a section across the Atlantic Basin can give some indication of the rate of trans-oceanic exchange relative to other veliger species taken from the same samples. As a first approximation the data from all stations in the North and Equatorial Atlantic can be pooled (Table VI). If the frequency of occurrence is expressed as the per cent of all stations at which a species was found and the ten species considered are accordingly ranked, then *Cymatium parthenopeum* will be at the top of the list, appearing in 26.9 per cent of the samples, whereas *Tonna maculosa* will be at the bottom, being found in only 0.6 per cent of the samples.

Cymatium parthenopeum is a species that as an adult is commonly found in the shelf fauna throughout the warm and tropical North Atlantic Ocean (Clench and Turner, 1957), whereas *Tonna maculosa* is principally found in the western Atlantic, being known only from a few scattered records in the eastern Atlantic (Fischer-Piette and Nicklès, 1946). One can perhaps conclude that *Tonna maculosa* is occasionally re-introduced into the eastern Atlantic and has never become established, or alternately, that it has been consistently overlooked in eastern Atlantic collections.

Notwithstanding its long pelagic larval development (Table I), *Cymatium nicobaricum* has an adult distribution somewhat similar to that of *Tonna maculosa*. In the eastern Atlantic it has been found only on the Madeira and Canary Islands (Clench and Turner, 1957; Odhner, 1931; Dautzenberg, 1890).

Regarding the remaining gastropod species one can ask whether the relative amount of isolation between their eastern and western Atlantic populations is reflected in the degree of morphological difference between them. The assumption is that the degree of difference is inversely related to the amount of genetic exchange between them and that the rate of gene-flow is related to the frequency with which larvae of a particular species are found relative to other species of veligers in the plankton. Hence, the fewer samples in which a veliger species is found, the greater the probability that populations on either side of the Atlantic will be represented by distinct subspecies.

The case of *Philippia krebsii* is problematic since the species is not easily collected as an adult and was not even known to occur in the eastern Atlantic until quite recently (Robertson, 1964; Robertson *et al.*, 1970).

The genus *Pedicularia* in the North Atlantic is often considered to include two species, one on either side of the Atlantic. These two forms are, however, probably best regarded as a single species, perhaps composed of two geographical subspecies. In any case not enough adult specimens are presently available for determining morphological variability of the eastern and western Atlantic forms.

Smaragdia viridis has two subspecies, *viridis* in the eastern Atlantic and *viridemaris* in the western Atlantic, although the frequency of its occurrence appears high on the list. However, 75 per cent of all specimens found in east-west trans-Atlantic currents occurred at the western end of the North Atlantic Drift (Fig. 11). Moreover, the length of its larval development probably limits the frequency of its dispersal (Table I).

Both *Charonia variegata* and *Tonna galea* are highly variable species. Neither are regarded as having geographic subspecies (Clench and Turner, 1957; Turner, 1946).

The percentage of stations at which *Phalium granulatum* is found is considerably less than the preceding two species. *Phalium granulatum* was recently reviewed by Abbott (1968), who considers it to have three subspecies, namely, *undulatum* in the eastern Atlantic and Mediterranean, *granulatum* in the western Atlantic including the West Indies, Central America, and Northern Brazil, and *centriquadratum* in the eastern Pacific from Southern California to Peru.

Near the bottom of the list and perhaps one of the most interesting examples is *Thais haemastoma.* This highly variable species is usually considered to have four subspecies: *haemastoma* and *forbesi* in the eastern Atlantic and *haemastoma, floridana,* and *haysae* in the western Atlantic (Fig. 9). The dispersal in the easterly direction is in the North Atlantic Drift, where it has been found only in the late summer and early fall. Butler (1954) observed that the species breeds in the Gulf of Mexico during June, July, and August. Hence, it is one of the few examples in which there appears to be some seasonality in the occurrence of larvae. Dispersal in a westerly direction is evidently restricted to the South Equatorial Current. Here the larvae have also been found in winter months.

The data on the frequency of larval occurrence may now be briefly summarized. The veliger larvae of *Cymatium parthenopeum, Charonia variegata,* and *Tonna galea* are common throughout the North Atlantic gyre and no geographic subspecies are recognized among these three forms. *Philippia krebsii* and *Pedicularia sicula* (*s.l.*) are both problematic because their geographic distribution and adult morphology have been insufficiently studied. *Smaragdia viridis* and *Phalium granulatum* have restricted larval dispersal and both have eastern and western Atlantic subspecies. *Thais haemastoma,* whose larvae occur even less widely in the open ocean, is regarded as having four subspecies. Finally, *Cymatium nicobaricum* and *Tonna maculosa* evidently are only very rarely dispersed and are known as adults from only a few records in the eastern Atlantic. Thus even with such a relatively crude measure, there seems to be a relationship between the morphological differences found in widely separated populations and the relative rate of genetic exchange by larval dispersal. Although the relationship is imperfect owing to a lack of knowledge in the variability of adult populations of tropical marine gastropods, a perfect relationship is not expected. If the characters transmitted by larvae are unfavorable in the new environment of the recipient population, selection will tend to eliminate their effect no matter what the rate of larval dispersal may be.

There remain many imponderables. Do the slight shell variations that one sees in geographical subspecies really offer any selective advantage? Possibly not, but in the only two genetic investigations published on marine gastropods where this problem has been studied, on *Purpura lapillus* by Staiger (1957) and the detailed study of Struhsaker (1968) on the tropical intertidal species *Littorina picta,* it is shown that differences in the thickness and sculpturing of the shell were related genetically to highly adaptive physiological characteristics. Thus Struhsaker showed that the sculpturing of *Littorina picta* was genetically related to growth rate and survival of the larvae and also to the resistance to dessication by the adult form.

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On January 21, 1971, Professor dr. phil. Gunnar Thorson passed on at the age of 64. He had yet much to offer and his untimely death was a great shock and a sincere loss. Gunnar Thorson's writings and great enthusiasm have been a constant source of inspiration since first I read his work in my student days. When last I saw him in Venice in October 1970, I gave him a manuscript copy of this paper not knowing that it was to be one of our last intellectual exchanges. It seems fitting therefore that I should dedicate this paper to the memory of Prof. Gunnar Thorson.

SUMMARY

1. Ten species of prosobranch gastropod veligers collected from the open waters of the North Atlantic Ocean have been identified by comparison of their larval shells with the protoconchs of identifiable juvenile or adult museum specimens. The larvae described are those of *Cymatium parthenopeum* (von Salis), *Cymatium nicobaricum* (Röding), and *Charonia variegata* (Lamarck) belonging to the family Cymatiidae; *Tonna galea* (Linné) and *Tonna maculosa* (Dillwyn) belonging to the family Tonnidae; *Phalium granulatum* (Born) belonging to the family Cassidae; *Thais haemastoma* (Linné), a muricid; *Philippia krebsii* (Mörch), an architectonicidae; *Smaragdia viridis* (Linné), a neritid; and *Pedicularia sicula* Swainson belonging to the family Ovulidae.

2. The geographical distribution of the veligers of these ten gastropod species has been determined in the North and tropical Atlantic from approximately eight hundred and fifty plankton tows. The relationship between the North and Equatorial Atlantic circulation and the dispersal of gastropod veliger larvae can be seen from these data (Figs. 5–12). *Charonia variegata, Philippia krebsii,* and *Pedicularia sicula* were found in all three trans-Atlantic currents sampled, namely, the eastwardly moving North Atlantic Drift and the westwardly flowing North and South Equatorial Current. *Cymatium parthenopeum* and *Tonna galea* were found throughout the North Atlantic gyre, but only from scattered records in the South Equatorial Current. *Phalium granulatum* and *Thais haemastoma* were found in the North Atlantic Drift and South Equatorial Current. These seven species are regularly dispersed in either direction across the North Atlantic barrier. *Smaragdia viridis* was found in the western half of the North Atlantic Drift and in the eastern half of the South Equatorial Current; it is probably less frequently transported across the Atlantic. *Cymatium nicobaricum* veligers were found only once in mid-ocean in the North Atlantic Drift; all other records were restricted to the tropical and warm temperate Western Atlantic. *Tonna maculosa* veligers were found only in the Gulf Stream. The adults of all ten species are amphi-Atlantic in their geographical distribution and occur in the tropical and

warm-temperate shelf waters. Adults of *Cymatium nicobaricum* and *Tonna maculosa,* however, are known from only a few records in the eastern tropical Atlantic.

3. The duration of pelagic larval development has been estimated for the same ten species of gastropods. Six species, *Charonia variegata, Cymatium parthenopeum, Cymatium nicobaricum, Tonna galea, Tonna maculosa,* and *Phalium granulatum* have a period of pelagic development of over three months. *Pedicularia sicula* and *Smaragdia viridis* can probably reach the settling state in less than two months. It is possible that the latter four forms have settling responses and can delay metamorphosis. A comparison between the duration of larval development and the velocity of the North and tropical Atlantic surface currents shows that transoceanic dispersal of the first six mentioned species is possible even without a delay in settlement.

4. The frequency of long-distance dispersal across ocean basins is chiefly dependent upon (a) the drift coefficient, that is, the probability that larvae will be carried off-shore into the major ocean surface currents rather than retained in the coastal waters of the parent population, and (b) the size of the parent population from which the larvae originate. There may be considerable variation in the frequency of long-distance dispersal related to larval mortality.

5. The lower limits of sensitivity obtained by using a conventional plankton net are such that it is possible for trans-oceanic dispersal to occur and yet go completely unnoticed. The concentration of only one larva per tow reoccurring at each station along a transect across the Atlantic may represent a significant amount of trans-oceanic dispersal.

6. If pelagic larvae are important in maintaining genetic continuity, then the degree of morphological differentiation between eastern and western Atlantic populations of gastropod species having amphi-Atlantic distributions would be expected to bear an inverse relationship to the frequency with which the veliger larvae of these species were found in the plankton of the open sea. The evidence from the gastropod species considered here seems to support this hypothesis (Table V).

LITERATURE CITED

Abbott, R. T., 1968. The helmet shells of the world (Cassidae). Part I. *Indo-Pac. Mollusca,* **2**(9): 15–201.

Allen, J., 1956. *Cowry Shells of World Seas.* Melbourne, Georgian House, 170 pp.

Bumpus, D., and L. M. Lauzier, 1965. Surface circulation on the continental shelf off eastern North America between Newfoundland and Florida. *Ser. Atlas of the Marine Environment* (Ed. Wilfred Webster) Folio 7, New York, *Amer. Geogr. Soc.* 4 pp., 8 pl.

Butler, P. A., 1954. The southern oyster drill. *Proc. Nat. Shellfish Ass.,* **1953**: 67–75.

Chesher, R. H., 1966. The R/V Pillsbury deep-sea biological expedition to the Gulf of Guinea, 1964–65. 10. Report on the Echinoidea collected by R/V Pillsbury in the Gulf of Guinea. *Stud. Trop. Oceanogr. Miami,* **4**(1): 209–223.

Clench, W. J., 1947. The genera *Purpura* and *Thais* in the Western Atlantic. *Johnsonia,* **2**: 61–92.

Clench, W. J., and R. D. Turner, 1957. The family Cymatiidae in the Western Atlantic. *Johnsonia,* **3**: 189–244.

Coe, W. R., 1956. Fluctuations in populations of littoral marine invertebrates. *J. Mar. Res.,* **15**(3): 212–232.

Craven, A. E., 1877. Monographie du genre *Sinusigera* d'Orb. *Ann. Soc. Malacol., Bruxelles*, **12**: 105–127.

Craven, A. E., 1883. On the genus *Sinusigera* d'Orbigny. *Ann. Mag. Natur. Hist. (Series 5)*, **11**: 141–142.

Dautezenberg, P., 1889. Contribution à la faune malacologique des Îles Açores. *Résult. Camp. Scient. Prince Albert I Fasc. I*, pages, 1–112, 4 pls.

Dautzenberg, P., 1890. Récoltes malacologiques de M. l'abbé Cullieret aux iles Canaries et au Sénégal en janvier et février 1890. *Mem. Soc. Zool. Fr.*, **1890**: 147–148.

Dawydoff, C., 1940. Quelques véligères géantes de Prosobranches provenant de la mer de Chine. *Bull. Biol. Fr. Belg.*, **74**(4): 497–507.

Dickson, C. N., and F. Evans, 1956. The *Petula's* meterological logbook. *Marine Observer*, (Oct.) **1956**: 215–218.

Ekman, Sven, 1953. *Zoogeography of the Sea.* Sidgwick and Jackson Limited, London, 417 pp.

Fischer-Piette, E., and M. Nicklès, 1946. Mollusques nouveaux ou peu connus des côtes de l'Afrique occidentale. *J. Conchyllol.*, **87**(2): 45.

Fretter, V., and A. Graham, 1962. *British Prosobranch Molluscs-Their Functional Anatomy and Ecology.* The Ray Society, London, 755 pp.

Fuglister, F. C., 1963. Gulf Stream "60." *Prog. Oceanogr.*, **1**: 263–373.

Guppy, H. B., 1917. *Plants, Seeds, and Currents in the East Indies and Azores.* William Norgate, London, 531 pp.

Hadfield, M. G., and R. H. Karlson, 1969. Externally induced metamorphosis in a marine gastropod. *Amer. Zool.*, **9**(4): 1122.

Hedley, C., 1903. Scientific results of the trawling expedition of H.M.C.S. "Thethis" off the coast of New South Wales in February and March 1898. Mollusca, Part II. Scaphopoda and Gastropoda. *Mem. Aust. Mus.*, **4**: 327–402.

Khanaychenko, H. K., N. Z. Khlystov and V. G. Zhidov, 1965. The system of equatorial countercurrents in the Atlantic Ocean. *Okeanologiya*, **5**: 222–229. (Translation: *Acad. Sci. USSR Oceanology, Scripta Technica*, pages 24–32.)

Kiseleva, G. A., 1966. [Investigations on the ecology of larvae of certain larger forms of benthic animals of the Black Sea.] Abstracts of dissertations for the candidate's degree in Biological Sciences. Odessa, Gosudarstvenuyy Universitet I. I. Mechnikova, Odessa, 20 pp. (In Russian.)

Kiseleva, G. A., 1967. [The effect of the substrate on settling and metamorphosis of larvae of benthic animals.] In: Donnyye Biotsendsy i Biologiya Bentoshykh Organismnov Chernogo Morya [Bottom Biocenoses and Biology of Benthic Organisms of the Black Sea] pages 71–84. (Respublikansky Mezhdom-Strennyy Sbornik, Ser. "Biologiya Morya" Kiev). (In Russian.)

Knudsen, J., 1950. Egg capsules and development of some marine prosobranchs from tropical west Africa. *Atlantide Rep.*, **1**: 85–130.

Knudsen, J., 1956. Marine prosobranchs of tropical west Africa (Stenoglossa). *Atlantide Rep.*, **4**: 9–110.

Laxton, J. H., 1969. Reproduction in some New Zealand Cyamtiidae (Gastropoda:Prosobranchia). *Zool. J. Linn. Soc.*, **48**: 237–253.

Lebour, M. V., 1932. The larval stages of *Simnia patula*. *J. Mar. Biol. Ass. U. K.*, **18**: 107–110.

Lebour, M. V., 1945. The eggs and larvae of some prosobranchs from Bermuda. *Proc. Zool. Soc. London*, **114**: 462–489.

Loosanoff, V. L., 1964. Variations in time and intensity of setting of the starfish, *Asterias forbesi*, in Long Island Sound during a twenty-five-year period. *Biol. Bull.*, **126**: 423–439.

Loosanoff, V. L., 1966. Time and intensity of setting of the oyster, *Crassostrea virginica*, in Long Island Sound. *Biol. Bull.*, **130**(2): 211–227.

MacDonald, J. D., 1858. On the probable metamorphosis of *Pedicularia* and other forms; affording presumptive evidence that the pelagic Gastropoda, so called, are not adult forms, but, as it were, the larvae of well-known genera, and perhaps confined to species living in deep water. *Trans. Linn. Soc. London*, **22**: 241–243.

Mann, C. R., 1967. The termination of the Gulf Stream and the beginning of the North Atlantic Current. *Deep-Sea Res.*, **14**: 337–359.

Marche-Marchad, I., 1969. Les Architectonicidae (Gastropoda: Prosobranches) de la côte occidentale d'Afrique. *Bulletin de l'Institut Fondamental d'Afrique Noire, Series A.*, **31**: 461–486.

Menzies, R. J., J. S. Zaneveld and R. M. Pratt, 1967. Transported turtle grass as a source of organic enrichment of abyssal sediments off North Carolina. *Deep-Sea Res.*, **14**: 111–112.

Metcalf, W. G., A. D. Voorhis and M. C. Stalcup, 1962. The Atlantic Equatorial Undercurrent. *J. Geophys. Res.*, **67**: 2499–2508.

Mileikovsky, S. A., 1966. Range of dispersion of the pelagic larvae of benthic invertebrates by currents and the migratory role of this dispersion taking Gastropoda and Lamellibranchia as examples. *Okeanologiya*, **6**: 482–492. (Translation: *Acad. Sci. USSR Oceanology, Scripta Technica*, pages 396–404.)

Millar, R. H., and J. M. Scott, 1967. The larva of the oyster *Ostrea edulis* during starvation. *J. Mar. Biol. Ass. U. K.*, **47**(3): 475–484.

Moore, D. R., 1961. The marine and brackish water mollusca of the State of Mississippi. *Gulf Res. Rep.*, **1**(1): 1–55.

Odhner, Nils H. J., 1931. Beiträge zur Malakozoologie der Kanarischen Inseln. *Ark. Zool.*, **23A**(14): 1–116.

Ranson, Gilbert, 1967. Les protoconques ou coquilles larvaires de Cyprées. Les Cyprées du Laboratoire de Malacologie du Muséum National d'Histoire Naturelle. *Mém. Mus. Nat. Hist. Natur., Paris Series A. Zool.*, **47**(2): 93–126.

Rinkel, M. O., S. Sund and G. Neuman, 1966. The location of the termination area of the equatorial undercurrent in the Gulf of Guinea based on observations during Equalant III. *J. Geophys. Res.*, **71**: 3893–3901.

Robertson, R., 1964. Dispersal and wastage of larval *Philippia krebsii* (Gastropoda: Architectonicidae) in the North Atlantic. *Proc. Acad. Nat. Sci. Philadelphia*, **116**: 1–27.

Robertson, R., 1967. *Heliacus* (Gastropoda: Archtectonicidae) symbiotic with Zoanthiniaria (Coelenterata). *Science*, **156**: 246–248.

Robertson, R., 1970a. Systematics of Indo-Pacific *Philippia* (*Psilaxis*), architectonicid gastropods with eggs and young in the umbilicus. *Pacif. Sci.*, **24**: 66–83.

Robertson, R., 1970b. Review of the predators and parasites of stony corals, with special reference to symbiotic prosobranch gastropods. *Pacif. Sci.*, **24**: 43–54.

Robertson, R., R. S. Scheltema and F. W. Adams, 1970. The feeding, larval dispersal and metamorphosis of *Philippia* (Gastropoda: Architectonicidae). *Pacif. Sci.*, **24**: 55–65.

Scheltema, R. S., 1956. The effect of substrate on the length of planktonic existence of *Nassarius obsoletus*. *Biol. Bull.*, **111**: 312.

Scheltema, R. S., 1961. Metamorphosis of the veliger larvae of *Nassarius obsoletus* (Gastropoda) in response to bottom sediment. *Biol. Bull.*, **120**: 92–109.

Scheltema, R. S., 1964. Origin and dispersal of invertebrate larvae in the North Atlantic. *Amer. Zool.*, **4**(3): 299–300.

Scheltma, R. S., 1966a. Evidence for trans-Atlantic transport of gastropod larvae belonging to the genus *Cymatium*. *Deep-Sea Res.*, **13**: 83–95.

Scheltma, R. S., 1966b. Trans-Atlantic dispersal of veliger larvae from shoal-water benthic mollusca. *Second Int. oceanogr. Congr.* (*Moscow*) *Abstr.*, No. **375**: 320.

Scheltema, R. S., 1967. The relationship of temperature to the larval development of *Nassarius obsoletus* (Gastropoda). *Biol. Bull.*, **132**: 253–265.

Scheltema, R. S., 1968. Dispersal of larvae by equatorial ocean currents and its importance to the zoogeography of shoal-water tropical species. *Nature*, **217**: 1159–1162.

Scheltema, R., in press. The dispersal of the larvae of shoal-water benthic invertebrate species over long distances by ocean currents. *Proc. Fourth European Symposium Mar. Biol.* (*Bangor, N. Wales*), Cambridge University Press.

Schumacher, A., 1940. Monatskarten der oberflächenströmungen im Nord Atlantischen Ozean (5°S bis 50°N). *Annalen der Hydrographie und Maritime Meterologie, Berlin*, **68**: 109–123.

Schumacher, A., 1943. Monatskarten der oberflächenströmungen im äquatorialen und Südlichen Atlantischen Ozean. *Annalen der Hydrographie und Maritime Meteorolgie Berlin*, **71**: 209–219.

Simpson, George Gaylord, 1952. Probabilities of dispersal in geologic time. *Bull. Amer. Mus. Natur. Hist.*, **99**: 163–176.

Simroth, H., 1895. *Die Gastropoden der Plankton-Expedition.* Ergn. Plankton-Exp. d. Humboldt-Stift. II, F., d, Verlag von Lipius und Tischer, Kiel und Leipzig, 206 pages.

Staiger, H., 1957. Genetical and morphological variation in *Purpura lapillus* with respect to local and regional differentiation of population groups. *Année Biol.*, **33**(3): 251–258.

Stalcup, M. C., and W. G. Metcalf, 1966. Direct measurements of the Atlantic Equatorial Undercurrent. *J. Mar. Res.*, **24**: 44–55.

Stander, G. H., L. V. Shannon and J. A. Campbell, 1969. Average velocities of some ocean currents as deduced from the recovery of plastic drift cards. *J. Mar. Res.*, **27**(3): 293–300.

Struhsaker, J. W., 1968. Selection mechanisms associated with intra-specific shell variation in *Littorina picta* (Prosobranchia:Mesogastropoda). *Evolution*, **22**(3): 459–480.

Strum, M., and K. Voigt, 1966. Observations on the structure of the equatorial undercurrent in the Gulf of Guinea in 1964. *J. Geophys. Res.*, **71**: 3105–3108.

Thiriot-Quiévreux, C., 1967. Observations sur le développement larvaire et postlarvaire de *Simnia spelta* Linné (Gastéropode:Cypraeidae). *Vie Milieu*, **18**(1 A): 143–151.

Thompson, T. E., 1958. The natural history, embryology, larval biology and post-larval development of *Adalaria proxima* (Alder and Hancock) (Gastropoda:Opisthobranchia). *Phil. Trans. Roy. Soc., London*, 242B: 1–58.

Thompson, T. E., 1962. Studies on the ontogeny of *Tritonia hombergi* Cuvier (Gastropoda:Opisthobranchia). *Phil. Trans. Roy. Soc., London*, 245B: 171–218.

Thorson, G., 1940. Studies on the egg masses and larval development of gastropoda from the Iranian Gulf. *Danish Scientific Investigations in Iran, Copenhagen*, pt. **2**: 159–238.

Thorson, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates. *Medd. Komm. Dan. Fisk. Havundersøg., Copenhagen, Series Plankton*, **4**(1): 1–523.

Thorson, G., 1961. Length of pelagic life in marine invertebrates as related to larval transport by ocean currents. Pages 455–474 in M. Sears, Ed., *Oceanography*, Pub. 67, American Association for the Advancement of Science, Washington, D. C.

Turner, R. D., 1946. The family Tonnidae in the Western Atlantic. *Johnsonia*, **2**(26): 165–192.

Turner, R. D., and A. C. Johnson, 1968. Biological studies in marine wood borers. *Annu. Rep. Amer. Malacological Union*, 1968: 14–16.

U. S. Hydrographic Office, 1967. *Pilot Charts.* U. S. Goverment Printing Office, Washington, D. C.

Voigt, K., 1961. Äquatoriale Unterströmung auch in Atlantik (Ergebnisse von Strömungsmessungen auf einer Atlantischen Ankerstation der "Michail Lomonossov" an Äquator im Mai 1959). *Beitraege Zur Meerskunde, Akademie der Wissenschaften, Berlin*, **1**: 56–60.

Voorhis, A. D., 1961. Evidence of an eastward equatorial undercurrent in the Atlantic from measurements of current shear. *Nature*, **191**: 157–158.

Wallace, A. R., 1876. *The Geographical Distribution of Animals, Volume I.* Harpers, New Yerk, 503 pp.

Worthington, L. V., 1962. Evidence for a two gyre circulation system in the North Atlantic. *Deep-Sea Res.*, **9**: 51–67.

Reference: *Biol. Bull.*, **140**: 323–330. (April, 1971)

INTRASPECIFIC VARIATION IN THE BRITTLE-STAR *OPHIOPHOLIS ACULEATA* (LINNAEUS) IN THE NORTHWESTERN ATLANTIC (ECHINODERMATA; OPHIUROIDEA)[1] [2]

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The genus *Ophiopholis* Müller and Troschel, 1842 includes several species of brittle-stars which display a considerable amount of intraspecific morphological variation. According to Clark (1911), separation of species within this genus solely on disk covering, number and arrangement of the naked aboral disk plates, length of the arm spines, shape of arm plates, and number, form and arrangement of the arm platelets, is futile. Variations in these and other characters customarily employed to distinguish ophiuroid species make it exceedingly difficult to determine reliable species characters within this genus. After examining over 5000 specimens of *Ophiopholis* from around the world, Clark (1911) concludes there are five extant species. He states *O. aculeata* is the "primary" species of the group, probably meaning it is the ancestral stock of a monophyletic group. *O. aculeata* has an Arctic—circumboreal distribution and extends southward to Japan and northern California in the North Pacific, and to Cape Hatteras and the English Channel in the North Atlantic. It normally inhabits moderate depths ranging from 0–300 meters, but a few specimens have been collected as deep as 1000 meters. Clark (1911) observed no great differences between the European and North American specimens of *O. aculeata* and none between those from the northern and southern extremes of its North American range.

A biometrical study of *O. aculeata* was undertaken to determine the morphological variability of the most ubiquitous species of this genus and to describe the differences in morphology occurring off the coasts of Maine, Newfoundland and Greenland.

Methods and Materials

Fourteen taxonomic characters were examined statistically for three samples of *O. aculeata*. Each sample contains 200 specimens with a normal distribution of disk diameters and was collected from depths of less than 600 meters. The southern sample is from off the coast of Maine (approximate Latitude, 44°N), the intermediate sample is from off the coast of Newfoundland (approximate Latitude, 48–50°N) and the northern sample is from off the coast of southwestern Greenland (approximate Latitude, 62–67°N). Disk diameter was measured to the nearest 1.0 mm with vernier calipers. Measurements of the oral shields, arm spines and disk spines were made with an ocular micrometer to the nearest 0.03 mm. The oral shield serving as the madreporite is considerably more rounded than the other oral shields and was not used. The longest arm spine on the sixth arm

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TABLE I

Continuous variable character comparisons between three populations of O. aculeata

Character	Populations compared*	Observed t-value**	Coefficient of difference	Significance
Width-to-length ratio of the oral shields	1 and 2	1.20	0.05	no
	1 and 3	−3.45	0.20	yes
	2 and 3	−4.47	0.24	yes
Length of the oral disk spines	1 and 2	9.06	0.47	yes
	1 and 3	5.95	0.30	yes
	2 and 3	−1.95	0.11	no
Length of the aboral disk spines	1 and 2	−0.53	0.03	no
	1 and 3	−1.76	0.09	no
	2 and 3	−1.37	0.07	no
Number of naked aboral disk plates	1 and 2	−9.12	0.46	yes
	1 and 3	−10.18	0.55	yes
	2 and 3	−1.34	0.07	no
Number of aboral arm platelets	1 and 2	7.28	0.37	yes
	1 and 3	3.32	0.17	yes
	2 and 3	−4.26	0.21	yes

* 1, Maine population; 2, Newfoundland population; 3, Greenland population.
** In all cases there are 398 degrees of freedom and 0.99t = 2.58.

segment was used for all arm spine measurements. The number of oral papillae was taken as the number occurring on five or more of the half jaws. The morphology of the oral and aboral disk spine tips was arbitrarily divided into two categories: smooth when they terminated in a rounded tip, and thorny when they terminated in a crown of thorns. The number of naked aboral disk plates does

TABLE II

Discrete variable character comparisons between three populations of O. aculeata

Character	Populations compared*	Observed chi-square	0.99 chi-square	Coefficient of difference	Significant
Number of oral papillae	1 and 2	2.79	6.63	0.09	no
	1 and 3	27.23	9.21	0.26	yes
	2 and 3	41.28	9.21	0.34	yes
Morphology of the oral disk spine tip	1 and 2	43.83	6.63	0.35	yes
	1 and 3	74.75	6.63	0.49	yes
	2 and 3	4.60	6.63	0.10	no
Morphology of the aboral disk spine tip	1 and 2	2.73	6.63	0.08	no
	1 and 3	0.00	6.63	0.00	no
	2 and 3	2.73	6.63	0.08	no
Number of arm spines	1 and 2	6.17	9.21	0.12	no
	1 and 3	3.61	9.21	0.08	no
	2 and 3	6.84	11.30	0.08	no
Number of naked radial shields	1 and 2	3.96	9.21	0.10	no
	1 and 3	1.24	9.21	0.02	no
	2 and 3	4.64	9.21	0.08	no
Presence of aboral arm platelet spines	1 and 2	0.25	6.63	0.03	no
	1 and 3	0.59	6.63	0.05	no
	2 and 3	0.00	6.63	0.01	no

* 1, Maine population; 2, Newfoundland population; 3, Greenland population.

not include the naked radial shields. The radial shields were arbitrarily divided into three categories: covered, when 8–10 were covered with spines; partially covered, when 3–7 were covered with spines; and naked, when 0–2 were covered with spines. The number of aboral arm platelets was taken as the number adjacent to the aboral arm plate of the sixth arm segment. The aboral arm platelet spines were considered either present or absent.

Sample means were compared by the t-test at the 0.99 level of confidence and sample frequencies were compared by the Chi-square test at the 0.99 level of confidence. The correlation coefficient (r) and the standard error of the regression line (S.E.) are given with all regression equations. All calculations were obtained by the use of a Fortran PDIFF program on the University of Maine IBM-360 computer.

Table III

Statistics for characters that differ significantly between populations of O. aculeata

Character	Population	Mean	Standard deviation	Range
Width-to-length ratio of the oral shield	Maine	1.76	0.19	1.3–2.6
	Newfoundland	1.74	0.20	1.1–2.5
	Greenland	1.84	0.22	1.3–2.6
Number of oral papillae	Maine	3.32	0.53	2–5
	Newfoundland	3.42	0.59	2–6
	Greenland	3.05	0.50	2–4
Length of the oral disk spines (mm)	Maine	0.37	0.16	0.14–0.98
	Newfoundland	0.25	0.09	0.07–0.49
	Greenland	0.28	0.15	0.07–0.77
Morphology of the oral disk spine tip; 1 = smooth; 2 = thorny	Maine	1.57	0.50	1–2
	Newfoundland	1.24	0.43	1–2
	Greenland	1.15	0.36	1–2
Number of naked aboral disk plates	Maine	31.58	12.63	0–84
	Newfoundland	44.77	16.08	12–105
	Greenland	47.01	17.30	10–133
Number of aboral arm platelets	Maine	16.31	2.57	9–26
	Newfoundland	14.70	1.80	10–22
	Greenland	15.53	2.11	12–23

Results

The oral surface of the disk

Mean length-to-width ratios of the oral shields (Tables I and III) are essentially the same in the Maine and Newfoundland samples but are significantly greater in the Greenland sample. This ratio ranges from 1.1–2.6 for the species, or from nearly circular to oval.

The mean number of oral papillae (Tables II and III) is essentially the same in the Maine and Newfoundland samples but is significantly less in the Greenland sample. The number of oral papillae increases with an increase in mean disk diameter (Table IV).

TABLE IV

Statistics for discretely variable characters of O. aculeata *that change with size**

Character	Class	N	Mean of disk diam. (mm)**	Standard deviation (mm)	Range (mm)
Number of arm spines	(4)	11	2.6	0.81	2–4
	(5)	203	7.4	2.43	2–14
	(6)	329	11.2	2.54	6–19
	(7)	57	13.8	2.35	10–19
Number of oral papillae	(2)	24	5.5	2.78	2–11
	(3)	403	9.7	3.20	2–19
	(4)	165	11.3	3.13	5–19
	(5)	6	13.7	2.34	10–17
	(6)	2	10.0	3.38	12–14
Aboral arm platelet spines	(present)	23	4.1	1.96	2–8
	(absent)	577	10.2	3.20	2–19

* All populations were considered together.
** The mean of the disk diameters was calculated for each class.

The mean length of the longest oral disk spines (Tables I and III) is essentially the same in the Newfoundland and Greenland samples but is significantly longer in the Maine sample. An increase in length of oral disk spines is correlated with an increase in disk diameter, but the correlation coefficients are low, varying from 0.64–0.69 for the three samples.

The morphology of the oral disk spine tips (Tables II and III) differs significantly between Maine and Newfoundland but is essentially the same in New-

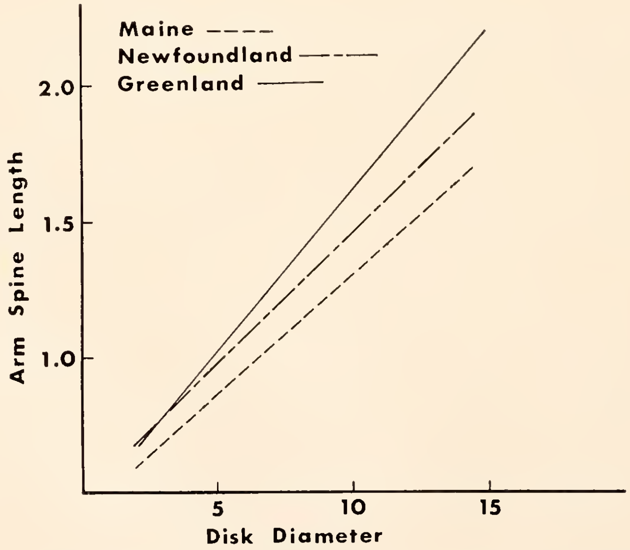

FIGURE 1. Linear regression lines for arm spine length as a function of disk diameter in the three populations of *O. aculeata*. All measurements are in millimeters.

foundland and Greenland. The frequency of specimens with thorny spine tips decreases clinally from 57–15 per cent, from Maine to Greenland.

The aboral surface of the disk

The mean length of the longest aboral disk spines (Table I) does not significantly differ between the three samples. An increase in spine length is correlated with an increase in disk diameter, but the correlation coefficients of the linear regression lines are low, ranging from 0.63–0.76.

The morphology of the aboral disk spine tip (Table II) does not significantly differ between the three samples. The frequency of specimens with thorny aboral spine tips ranges from 4–8 per cent for the three samples.

The number of naked radial shields is not significantly different in the three samples (Table II). Specimens with covered radial shields comprise 62.5 per cent of the combined samples, those with naked shields comprise 29.0 per cent and those with partially covered radial shields comprise 8.5 per cent of the combined samples.

The mean number of naked aboral disk plates (Tables I and III) increases along a cline from 32 in the Maine sample to 47 in the Greenland sample. This number is not correlated with disk diameter.

The arms

The mean number of aboral arm platelets (Tables I and III) differs significantly between the three samples, but does not vary clinally. The Maine sample has the largest mean of 16.3 and the Newfoundland sample has the smallest mean of 14.7. The Greenland sample is intermediate with a mean of 15.5 platelets.

There is no significant difference between the samples for the presence of aboral arm platelet spines (Table II). Platelet spines are present only on small specimens (Table IV) with a disk diameter of 8 mm or less. The spines do not occur on all small specimens since individuals as small as 2 mm were without platelet spines.

The frequencies for the number of arm spines (Table II) are essentially the same in all the samples. The number of arm spines increases from 4–7 with an increase in mean disk diameter from 2.6–13.8 mm (Table IV).

The length of the arm spines increases linearly with an increase in disk diameter (Fig. 1). The arm spines increase clinally in relative length from the Maine to the Greenland sample. The slope for the Greenland sample is significantly greater than the slopes of the more southern samples. Even though the slopes for the Maine and Newfoundland samples are not significantly different, the y-intercept is significantly greater in the latter, resulting in proportionately shorter arm spines in the Maine sample. The regression equations are:

Maine	$y = 0.90x + 0.44,\ r = 0.86,\ \text{S.E.} = \pm 0.13$ mm
Newfoundland	$y = 0.96x + 0.50,\ r = 0.86,\ \text{S.E.} = \pm 0.13$ mm
Greenland	$y = 1.20x + 0.41,\ r = 0.84,\ \text{S.E.} = \pm 0.19$ mm

y = arm spine length (mm)
x = disk diameter (mm)

The width of the arm spines increases linearly with an increase in disk diameter. The slopes and y-intercepts of the regression line do not differ significantly between the three samples. The regression equation for the combined sample is:

$$y = 0.81x - 0.01,\ r = 0.93,\ \text{S.E.} = \pm 0.07\ \text{mm}$$

y = arm spine width (mm)
x = disk diameter (mm)

The width of the arm spines increases linearly with an increase in arm spine length (Fig. 2). As shown above, arm spine length increases clinally with increase in latitude, but the width remains constant. This results in the arm spines being more acute in the more northern latitudes. Slopes for the linear regression lines differ significantly between the three samples. The regression equations are:

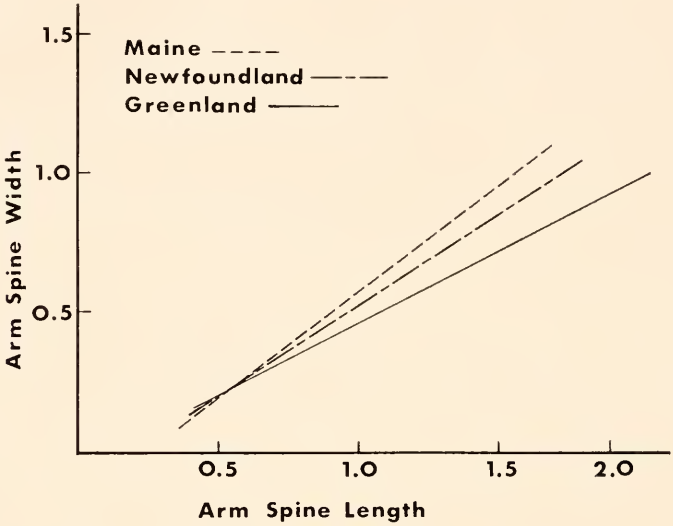

FIGURE 2. Linear regression lines for arm spine width as a function of arm spine length for the three populations of *O. aculeata*. All measurements are in millimeters.

Maine $y = 0.72x - 0.21,\ r = 0.89,\ \text{S.E.} = \pm 0.11$ mm
Newfoundland $y = 0.63x - 0.16,\ r = 0.80,\ \text{S.E.} = \pm 0.21$ mm
Greenland $y = 0.49x - 0.09,\ r = 0.83,\ \text{S.E.} = \pm 0.19$ mm
y = arm spine width (mm)
x = arm spine length (mm)

DISCUSSION

Characters that change clinally include (1) number of naked aboral disk plates, (2) length of the arm spines, and (3) length-to-width ratio of the arm spines.

Characters that are essentially the same in Maine and Newfoundland, but are distinctly different in Greenland include (1) length-to-width ratio of the oral shields, and (2) number of oral papillae.

Characters that are essentially the same in Newfoundland and Greenland, but are distinctly different in Maine include (1) length of the oral disk spines, and (2) morphology of the oral disk spine tip.

A character that does not change clinally but is distinctly different in all populations is the number of aboral arm platelets.

Characters that are essentially the same in all populations include (1) morphology of the aboral disk spine tip, (2) number of naked radial shields, (3) presence of aboral arm platelet spines, (4) length of the aboral disk spines, (5) width of the arm spines, and (6) number of arm spines.

None of the above characters are sufficiently different between samples that their coefficient of difference approaches the 1.28 value suggested by Mayr (1969) to be a basis for subspecific distinction. The magnitude of the differences is large enough to indicate the samples came from three phenotypically distinct populations. The uniformity of some characters in all the populations may indicate the expression of these characters is not significantly altered by environmental factors, such as temperature or salinity. Those characters that vary clinally between populations are probably under the influence of some environmental parameter.

Two meristic ranges given for *O. aculeata* by previous workers, are too small. Mortensen (1927) and Dyakonov (1954) state there are 3 (rarely 2) oral papillae in *O. aculeata*. The present study shows there are 2–6 oral papillae with most specimens having 3–4. The number of arm spines ranges from 6–7, according to Mortensen (1927), and 5–7 according to Dyakonov (1954). The present study shows a range of 4–8. None of the specimens used in this study had more than 7 arm spines; however, some very large specimens, 22 mm in disk diameter or greater, had 8 arm spines on the sixth arm segment. Because the number of oral papillae and number of arm spines is size dependent, expansions in these ranges are expected when examining extremely small or large specimens.

Grieg (1893), cited in Mortensen (1933), states that Greenland specimens of *O. aculeata* are more spiny on the disk than ones from the Norwegian coast. According to Mortensen (1933), a large percentage of the Greenland specimens collected during the Danish Ingolf-Expedition had long spines on the disk. These spines were most prominent along the borders of the radial shields. He noted that they were particularly evident on specimens from the more northern localities with negative bottom temperatures. Clark (1911) notes that Alaskan specimens of *O. aculeata* have a more spinous disk than ones from Maine. This study indicates the oral disk spines of the Maine sample are significantly longer than those of the Newfoundland or Greenland samples and the aboral spine length is essentially the same in all three samples. A few specimens in the United States National Museum, Washington, D. C. and the Museum of Comparative Zoology, Harvard University, collected in the high Arctic have very long aboral disk spines, particularly around the radial shields. If the aboral disk spines are significantly longer in the specimens from the high Arctic, then the increase in length must occur somewhere north of the northernmost sampling area used in this study (approximate Latitude, 67°N).

Dyakonov (1954) described *O. pilosa* from the Sea of Okhotsk. This species is supposed to differ from *O. aculeata* in the following ways: 1) Short spinelets occur along the edge of the disk and terminate in a sharp thorn or crown of thorns;

2) The aboral arm platelets are numerous, small, rounded, and usually possess a spine; 3) The radial shields are naked. All these characters fall well within the range of variation displayed by *O. aculeata*. This makes the validity of *O. pilosa* questionable. A reevaluation of *O. pilosa* will probably indicate it is a junior synonym of *O. aculeata*.

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SUMMARY

1. Fourteen taxonomic characters were analyzed separately for Maine, Newfoundland and Greenland populations of *O. aculeata*.

2. The number of naked aboral disk plates, length of the arm spines and length-to-width ratio of the arm spines are characters that vary clinally.

3. The length-to-width ratio of the oral shields and the number of oral papillae are essentially the same in the Maine and Newfoundland populations but are significantly different in the Greenland population.

4. The length of the oral disk spines and morphology of the oral disk spine tip are essentially the same in the Newfoundland and Greenland populations but are significantly different in the Maine population.

5. The number of aboral arm platelets does not change clinally but is distinctly different in all populations.

6. The morphology of the oral disk spine tip, number of naked radial shields, presence of aboral arm platelet spines, length of the aboral disk spines, width of the arm spines and number of arm spines are characters essentially the same in all populations.

7. There are 2–6 oral papillae and 4–8 proximal arm spines present on *O. aculeata*.

8. The diagnostic characters of *O. pilosa* Dyakonov, 1954, fall within the range of variation displayed by *O. aculeata*.

LITERATURE CITED

CLARK, H. L., 1911. North Pacific ophiurans in the collection of the United States National Museum. *U. S. Nat. Mus. Bull.*, **75**: 1–302.

DYAKONOV, A. M., 1954. *Ophiuroids of the USSR Seas.* Izdatel'stvo Akademii Nauk SSSR Moskva-Leningrad, 131 p. [Translated from Russian, 1967, by the Israel Program for Scientific Translations, Jerusalem, TT 67-51490.]

GRIEG, J. A., 1893. Ophiuroidea. *Norske Nordhavs Expedition 1876–1878, Zool. (Christiania)*, **23**: 1–41.

MAYR, E., 1969. *Principals of Systematic Zoology.* McGraw-Hill Book Company, New York, 428 pp.

MORTENSEN, T., 1927. *Handbook of the Echinoderms of the British Isles.* Oxford University Press, New York, 421 pp.

MORTENSEN, T., 1933. Ophiuroidea. *Danish Ingolf-Expedition,* 4(8): 1–121.

Reference: *Biol. Bull.*, **140**: 331–338. (April, 1971)

ASPECTS OF MOLTING AND CALCIFICATION IN THE OSTRACOD *HETEROCYPRIS*

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The ostracod is a marine or freshwater crustacean with a bivalved carapace that encloses the rest of the body. The valves composing the carapace are heavily calcified and, being an integral part of the exoskeleton, they are shed during each molt. The valves of the new exoskeleton are then calcified while the rest of the exoskeleton remains unmineralized.

The calcification process associated with molting in ostracods is not well known. X-ray diffraction studies by Kesling (1951) on *Cypridopsis vidua* determined that the calcium is deposited within the ostracod valve as calcite. Fassbinder (1912), experimenting with the same species, concluded that the immediate source of the calcium used in calcification was from the body of the ostracod rather than from the aqueous environment. This was determined by growing the ostracods in calcium-free water and feeding them calcium-free food, and finding that they could still calcify the new valves after a molt. Van Morkhoven (1962) reported that the animal's diet will affect the calcification of the valves, with rich calcium diets producing secondary thickening and numerous protuberances. Finally, Kesling (1951) proposed that *C. vidua* has no special cells which secrete the shell material; it is formed rather by all the epidermal cells within the duplicature. He believed the ostracod calcification process was different from that of other crustaceans that have been studied.

The objective of this study was to gain some information about this calcification process. Experiments using Ca^{45} as a tracer were conducted to determine whether any calcium is reabsorbed from the valves prior to molting and whether calcium is stored for use in later valve calcification. Autoradiographs were prepared to determine which cells were active in calcifying the new valves.

MATERIALS AND METHODS

Cultures of *Heterocypris* were started from individuals collected at ponds in Cherry Hills Village, Colorado. These cultures were maintained in "Chlorofree" (Lambert-Kay Inc., Los Angeles) treated tap water at a pH of 7.4. Parboiled rice grains and hay were added as food. Isolated individuals were maintained in small culture dishes containing 22 ml treated tap water at pH 7.4, a one-quarter-inch piece of hay, one-quarter of a rice grain, one drop of detritus from the stock cultures, and some ground calcite to provide a rough substrate. Cultures were maintained at approximately 23° C.

In all the tracer studies the dosage of Ca^{45} (Nuclear Chicago) was one microcurie of the isotope per twenty-two ml of treated tap water. Determination of radioactivity was made by counting the organisms in metal planchets using a

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Nuclear Chicago model 186 decade scaler and a Geiger-Muller tube with a window thickness of 1.4 mg/cm^2.

Cytological and autoradiographic material was prepared by fixing in 5% formalin adjusted to pH 8.0 with NaOH, dehydrating with graded ethyl alcohols, and embedding in Epon 812. The embedded organisms were decalcified by nicking the anterior end of the Epon block to expose the valves, placing the blocks in 1 *N* HCl for one hour, and re-embedding in Epon 812 in a vacuum oven. This technique protected the soft tissues from acid degradation and prevented the loss of cellular calcium.

The material was sectioned at two microns on a Sorvall Mt-1 ultramicrotome. Sections were collected on distilled water at pH 8.0 and mounted on glass slides without any subbing. Sections were stained with either standard hematoxylin 5 minutes at 60° C and Saffranin-0 0.1% for 3 minutes at 60° C, or fast green FCF 0.1%, pH 2.0 at 60° C for five minutes, or toluidine blue (Trump, Smuckler and Benditt, 1961) modified by staining in Columbia staining jars for thirty minutes at 60° C.

Sections of specimens for autoradiography were fixed, embedded, and sectioned as above. Two-micron sections were mounted on slides, dipped in Kodak NTB-2 Nuclear Track Emulsion, and exposed for fourteen days in light-tight boxes. After development they were stained with toluidine blue, dehydrated, and mounted under coverslips.

The mineralogical composition of the valves was determined by X-ray diffraction of powdered valves mounted in a Debye-Scherrer powder camera.

Experimental Procedures

Determination of instar times

Three groups of fifteen animals each were isolated and observed hourly from the time of hatching until the adult stage was reached. The occurrence of each molt and the general condition of each animal was recorded.

Determination of calcium reabsorption

Twenty cultures were established, each containing 22 ml treated tap water and one microcurie Ca^{45}. Ten cultures contained ten animals each; ten cultures contained one animal each. The animals were allowed to molt in the radioactive media. After molting and calcification of the new valves, the animals were rinsed three times in treated tap water and transferred to identical cultures without Ca^{45}. The animals were then allowed to molt in the non-radioactive media, and the discarded valves and the animals were collected, washed, and dried for radioactivity assay.

Controls were prepared by placing ten dead ostracods killed by drying in an identical culture of Ca^{45} for the duration of the experiment. Additional controls were prepared by collecting the discarded non-radioactive valves from the first molt and exposing them to cultures containing Ca^{45} for the time period of the experiment.

Determination of calcium storage prior to molt

Thirty animals in the seventh instar were isolated in individual cultures containing one microcurie of Ca^{45}. The animals were allowed to undergo one complete molting cycle in this medium. At this stage of development the next molt normally occurs at 72 hours; therefore ten animals were removed 24 hours after molting in Ca^{45}, ten after 48 hours, and ten after 72 hours. Each group of organisms was dried on metal planchets after rinsing them three times in treated tap water.

Controls were established by exposing dead animals to the same concentration of Ca^{45} for 24, 48, and 72 hour intervals.

Observations and Results

As observed in our cultures, an ostracod beginning to molt can be distinguished by the development of clear areas along the ventral margins of the valves where they are starting to separate. During this time there is also a slight ballooning of the animal that causes it to lose its oval shape and to appear more rounded. As this swelling increases, swimming movements become erratic and the edges of the valves are rubbed against the bottom. This activity is interrupted by frequent periods of motionlessness.

Table I

Instar time periods under laboratory culture conditions. Molting occurs every one to three days depending on the stage of development attained by the individual

Instar	No. of animals observed	Time in hours between molts		
		Shortest	Longest	Mean
1st to 2nd	45	24	24	24
2nd to 3rd	45	24	48	26
3rd to 4th	45	24	48	29
4th to 5th	39	24	48	43
5th to 6th	36	24	72	53
6th to 7th	34	48	72	62
7th to 8th	34	48	96	74
8th to 9th	31	72	130	77

The actual discarding of the exoskeleton begins with the scraping of the inner surface of the valves by the thoracic legs followed by the withdrawal of the two pairs of antennae from the old exoskeleton and then the withdrawal of the remaining appendages. Once all the appendages are free, there is a further swelling of the ostracod that causes the old exoskeleton to separate completely from the body. The newly emerged individual appears soft and swollen. The transparent, uncalcified valves are gradually calcified over a period of several hours becoming opaque as calcification is completed.

Determination of instar times

Under laboratory culture conditions molting occurs every one to three days depending on the stage of development of the individual. As may be seen in Table I the younger instars molt more rapidly than the more mature animals and there is considerable individual variation in instar duration.

Determination of calcium reabsorption

Ostracods placed in water containing Ca^{45} do not incorporate Ca^{45} into the valves until the next molt and the calcification of the new valves. If the ostracods are then transferred to non-radioactive water, during the following molt the radioactive valves are shed, and none of the Ca^{45} in them is reabsorbed for use in calcifying the new valves. All the animals (110 individuals) that molted in Ca^{45}-containing water incorporated Ca^{45} into the valves as was shown by radioactive assay of the valves after they had been discarded during the following molt. The paired valves registered approximately 400 to 500 cpm. The ostracods and their new valves (108 individuals), when assayed by drying the whole animal on a planchet and then counting, contained no Ca^{45}. Thus, there was no reabsorption of Ca^{45} from the old valves prior to molting that was retained and used for calcifying the new valves. There was no uptake of Ca^{45} in either the dead control animals or in the valves discarded during the first molt in Ca^{45} and left in the containers for the duration of the experiment.

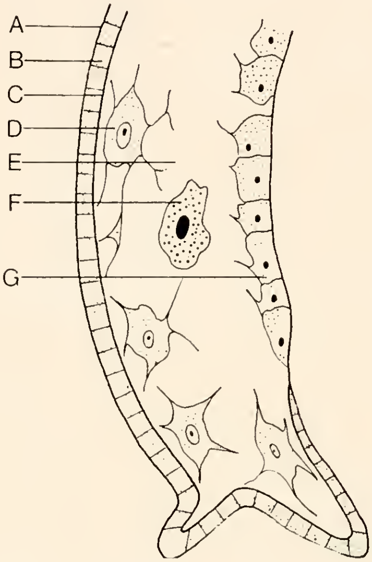

FIGURE 1. A simplified diagram of a section through the ventral margin of the duplicature: A, Outer chitinous layer; B, Calcified valve; C, Inner chitinous layer; D, Cell of outer lamella; E, Interlamellar space; F, Sub-dermal cell; G, Cell of inner lamella.

Determination of calcium storage

The ostracods used in this experiment were allowed to molt in a medium containing Ca^{45}. Therefore, the valves calcified during this molt contained Ca^{45} and had counts (400–500 cpm) equivalent to the radioactive valves recovered in the calcium reabsorption experiment above. Any significant storage of calcium in the tissues of the ostracod should be reflected in a higher cpm as the time of the next molt approached. The ostracods were kept in the Ca^{45} medium for 24, 48, and 72 hours after molting to determine whether there would be an additional Ca^{45} buildup that could represent calcium stored for the following molt and subsequent valve calcification.

In the ten ostracods exposed to Ca^{45} for 24 hours after molting there was a range from 403 to 536 cpm with a mean of 482 cpm. In the 10 exposed for 48 hours after molting there was a range from 437 to 534 cpm with a mean of 486 cpm and in the 10 exposed for 72 hours after molting there was a range from 448 to 515 cpm with a mean of 485 cpm. The controls only registered background. With the exception of 4 counts all the numbers lie within the 95% probability level for the deviation of the mean, indicating that there is no significant variation in the observed counts. Therefore, these results indicate that there is no buildup or storage of environmental calcium within the body of the animal prior to the molt.

TABLE II

Results of autoradiography showing time of calcification during molting process. During specimen preparation the valves were decalcified. Thus, the autoradiographs show only the Ca^{45} present in the cells active in calcification (the cells of the outer lamella) and not the increasing accumulation of Ca^{45} in the valves. At the end of seven hours the valves are calcified and the amount of Ca^{45} detectable in the cells of the outer lamella drops to zero.

Time after molting	Number of sections observed (animals)	Number of sections showing incorporation of Ca^{45}
5 minutes	120 (3)	0
30 minutes	160 (4)	160
1 hour	80 (2)	80
2 hours	120 (3)	80
3 hours	200 (5)	110
6 hours	160 (4)	90
7 hours	160 (4)	90
8 hours	80 (2)	0
11 hours	80 (2)	0
14 hours	80 (2)	0
18 hours	80 (2)	0

Histology

The general histological features of the duplicature of *Heterocypris* as determined by examination of sections stained with either haematoxylin or fast green are presented in Figure 1. The cells of the outer lamella are large, irregular in shape, and have processes extending outward to the valve and inward into the space between the outer and inner lamellae. These cells appear to be amoeboid and, during calcification, their positions within the lamella appear to change so they are found concentrated in the area being calcified. In the early stages of calcification they are concentrated at the margins of the valve, and, as these portions are calcified, they move in toward the mid-region which is the last part of the valve to be calcified.

The cells of the inner lamella are smaller in size and more regular in shape. Instead of being a loose aggregation of cells, they are organized into a more closely packed epithelium. Although the cells have small processes extending into the interlamellar space, they do not have an amoeboid appearance. The cells have fewer nucleoli than those of the outer lamella, and they stain less intensely both with fast green used as a general protein stain and with toluidine blue.

Autoradiography of sectioned material

Calcification of the valves starts within thirty minutes after the molt, and the cellular activity associated with calcification is completed within seven hours (Table II). With the cessation of this activity there is no further change in the appearance of the valves, and calcification has evidently been completed. The autoradiographs show a localization of the isotope only in the cells of the outer lamella. They also show that calcification starts at the margins of the valves and gradually proceeds toward the mid-region where calcification is finally completed.

Mineral composition of valves

The valves are mineralized exclusively with calcite.

Discussion

Several conclusions may be drawn from the results presented here. The first is that calcium dissolved in the water is the source of calcium for calcifying the valves of *Heterocypris*, although the incorporation pathways are unknown. Second, the animal does not reabsorb calcium from the old valves prior to the molt, and it does not build up a major store of calcium from other sources preparatory to molting. Third, calcification of the new valves is completed within seven hours after the molt and follows a pattern from the dorsal and ventral edges medially and from the anterior to the posterior of the animal. Finally, the cells of the outer lamellae are responsible for the deposition of calcite in the valves.

The Ca^{45} added to the culture water was incorporated into the new ostracod valves during calcification following the next molt. Whether the Ca^{45} was first incorporated into the food by action of the bacterial and fungal microfauna associated with the rice grains and was then ingested during feeding or whether it was directly absorbed by the cells of the body is not positively known. However, we favor the latter because: (1.) with no discernible method of calcium storage the animal would have to eat and digest a tremendous amount of food at the proper time to provide the large amounts of calcium necessary for the valves, (2.) in the many autoradiographs of *Heterocypris* used in this study there was no localization of Ca^{45} in the gut region or in the food it contained and (3.) the only cellular concentration of Ca^{45} found during the entire calcification process was in the cells of the outer lamella.

Van Morkhoven (1962) has reported that specimens of *Cypris pubera*, when fed on a diet of crushed snails rich in calcium, developed secondary thickening of the valves. It would appear from this that in *C. pubera* there is an assimilation of calcium from the food source. There could also have been an enrichment of the water in the culture by the dissolution of some of the crushed snail shell. Our attempts to duplicate this experiment with *Heterocypris* failed because the ostracods would not eat crushed snails taken from their native pond nor would they eat any other kind of meat offered to them. Perhaps a difference in dietary requirements reflects a difference in calcification mechanisms.

Our finding that calcium is neither stored nor reabsorbed by *Heterocypris* is also different from Fassbinder's (1912) report that in *C. vidua* the immediate source of calcium for valve calcification is calcium stored in the body at least five days prior to molting. This was shown by culturing the ostracods for that period of time in calcium-free water with calcium-free food and finding they could still calcify their new valves after molting. Although we believe that modern tracer methods are more reliable than the methods available to Fassbinder and that this difference could account for our divergent results, there may be alternative explanations. The possibility exists that under normal conditions of adequate available environmental calcium an ostracod does not store calcium in its tissues or reabsorb calcium from the old valves prior to molting. However, under conditions of calcium stress when environmental calcium is not available, calcium may be withdrawn from the old valves and stored for calcification of the new ones.

If the calcium of the valves is not reused, and it was not in our experiments, the problem becomes one of calcium storage. In some highly ornamented benthic species the valves must weigh several times as much as the weight of the organic material of the body. Where and in what form could this amount of calcium be stored? The presence of gastroliths, such as are found in the decapod crustaceans, is doubtful, and considering the small size of the digestive tract, it is doubtful that if they were present, they could store the amount of calcium needed for valve calcification. For these reasons it is our opinion that *Heterocypris* and probably most ostracods have not evolved the calcium storage mechanisms of the decapod crustacea, but instead have evolved cellular mechanisms for the rapid concentration and deposition of this element.

We believe that the cells of the outer lamella are responsible for calcifying the valves because they are in contact with the calcified valves by means of their numerous processes, because they are concentrated in the region of active calcification, and because they are the only cells to show Ca^{45} incorporation in autoradiographs. The lack of Ca^{45} in any other cells of the body during the entire calcification process leads to the tentative conclusion that the calcium is not transported through the body of the animal but is directly concentrated from the water by the cells of the outer lamella and transported intracellularly through the cell processes to the site of deposition in the valve.

In his study of *C. vidua* Kesling found no morphological differences between the cells of the inner and outer lamella although he postulated a functional difference for the two. If he had prepared a sequence of sections of animals at various periods of the molting cycle, perhaps morphological differences would have become apparent. In *Heterocypris* sp. the differences are marked during this time, and need further study. There appears to be an increase in the number of cells of both lamellae as the time of molting approaches, and during the calcification phase a movement of cells of the outer lamella occurs as explained above. Some of the changes are related to the production of mucopolysaccharides for the new exoskeleton and probably for the elaboration of the enzymes utilized in the molt. We have stained sections taken from animals at various time intervals during the molting cycle with the PAS reaction and have noted a marked increase in the amount of polysaccharides in the duplicature just before molting, but this needs more study before the complexities of the process are understood.

SUMMARY

Observations on living ostracods (*Heterocypris*) show that instars one to four require one to two days between molts, and instars five to eight require two to three days between molts.

Tracer experiments with Ca^{45} show that during the molting cycle the animal neither reabsorbs Ca from the old valves prior to molt nor stores Ca preparatory to molt.

Autoradiographs indicate that calcification of the valves occurs within seven hours after the molt and proceeds from the dorsal and ventral edges towards the valve center. The autoradiographs and other stained preparations indicate that the amoeboid-like cells of the outer lamella elaborate the calcite portion of the exoskeleton.

LITERATURE CITED

FASSBINDER, K., 1912. Bietrage zur Kenntnis der Susswasserostracoden. *Zool. Jahrb. Abt. Anat. Ont. Tiere,* **32**: 533–576.

KESLING, R. V., 1951. The morphology of ostracod molt stages. *Illinois Biol. Monogr.,* **21**(1–3): 1–126.

TRUMP, B. F., E. A. SMUCKLER, AND E. P. BENDITT, 1961. A method for staining epoxy sections for light microscopy. *J. Ultrastruct. Res.,* **5**: 343–348.

VAN MORKHOVEN, F. P. C. M., 1962. *Post Paleozoic Ostracoda: Their Morphology, Taxonomy, and Economic Use, Volume 1.* Elsevier Publishing Co., Amsterdam, London, New York, 204 pp.

Reference: *Biol. Bull.*, 140: 339–351. (April, 1971)

DELAY AND QUADRIPARTITION IN SEA URCHIN EGGS INDUCED BY SHORT EXPOSURE TO 2-MERCAPTOETHANOL

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The effects of 2-mercaptoethanol (ME), an inhibitor of cell division in a variety of cells (Mazia, 1958; Limbosch-Rolin and Brachet, 1961; Fautrez and Fautrez-Firlefyn, 1963; Fotakis, 1963; Limbosch-Rolin, 1963; Meeker, 1964; Mazia and Zeuthen, 1966), have been studied extensively by Mazia and his co-workers using echinoderm eggs. They reported that ME blocks cleavage reversibly if treatment is initiated before the "point of no return" which corresponds approximately to metaphase, and that the delay in cleavage is equal to the time spent in ME (Mazia, 1958; Mazia and Zimmerman, 1958). In addition, they found that DNA synthesis continues (Bucher and Mazia, 1960) and that the level of respiration is high enough to support cleavage (Mazia and Zimmerman, 1958) during the ME treatment. The mitotic apparatus (MA) can be isolated during the ME treatment, although the fibrous structures of MA seem disrupted with phase contrast microscopy (Mazia and Zimmerman, 1958). Pressure studies of Zimmerman (1964) revealed that ME also weakens cortical gel strength.

It was concluded that ME arrests cleavage at the time of exposure to ME, because the delay is equal to the duration of the treatment, and that this arrest is somehow linked to the abnormality in structure of the isolated MA.

In addition to the synthesis of DNA and ATP, the first cleavage of echinoderm eggs appears to require protein synthesis (Hultin, 1961; Zotin, Milman and Faustov, 1965; Young, Hendler and Karnofsky, 1969). In order to further elucidate the mechanisms of ME action on cleavage, it is necessary to obtain more detailed information on the response of cells to ME during the cleavage cycles and on the relationship between protein synthesis required for division and the effects of ME. These problems have been investigated in the present study.

The phenomenon of quadripartition (QP), a direct division of one cell into four, occurs during recovery from the prolonged blockage of cleavage by ME. QP involves the generation of a tetrapolar mitotic apparatus from a bipolar one (Mazia and Zimmerman, 1958). Mazia, Harris and Bibring (1960) established that each mitotic center is duplex in nature, *i.e.*, there are four potential centers at metaphase, and that duplication of centers occurs at about telophase. They further suggested that ME is capable of blocking duplication of the centers while permitting the "moving-apart" of existing centers according to the regular mitotic schedule. These conclusions were based on several observations. First, the quadripartitioned cells skipped the next cleavage although other mitotic events including the formation of a monopolar mitotic spindle occurred. Secondly, QP could not be induced by the ME treatment unless a certain time had elapsed after fertilization and unless exposure to ME was of such duration that it extended past the division of controls and the ME treated cells divided after the second division of controls. The inhibition of duplication of mitotic centers by ME was verified by Went (1966) using a more unambiguous agent (benzimidazole) for assaying the number of mitotic centers.

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In the present study, new observations have been made that may modify the present hypothesis regarding QP. The capacity of androgenic merogones to undergo QP was also examined.

MATERIALS AND METHODS

Arbacia punctulata eggs, obtained by electrical stimulation, were shed directly into a beaker filled with filtered sea water (Harvey, 1952). *Lytechinus variegatus* eggs were shed by placing a few drops of 0.55 M KCl into the test (Palmer, 1937). All eggs were washed twice.

The following procedures were used for the short exposure experiments. Eggs were inseminated and washed about a minute later to remove excess sperm. At 3–5 minute intervals after insemination, 1 ml of egg suspension, containing 400–500 eggs, was mixed with 1 ml of 0.2 M ME dissolved in sea water. After the desired period of exposure (5–80 minutes depending upon the experiments), eggs were washed by centrifugation or by dilution (a dilution factor of 1:10,000). Both centrifugation and dilution methods gave essentially the same results. The controls were treated similarly. The time when approximately 50% of the eggs had cleavage furrows was estimated visually. In order to determine the percentage of quadripartition (QP), 200 cells were examined before the onset of the second cleavage. Data obtained from asynchronously dividing cells were discarded.

After several preliminary experiments, O.1 M ME was chosen because this was the lowest concentration that gave consistent results. For example, 0.7 M did not block cleavage consistently in both *Lytechinus* and *Arbacia* eggs.

In some experiments ME-treated eggs were further treated with puromycin, a specific inhibitor of protein synthesis (Nathans, 1964). Each aliquot of eggs was quickly washed twice by dilution (for 1 minute) and 0.2 ml of egg suspension containing about 200 eggs was mixed with 0.2 ml of 1×10^{-3} M puromycin on a depression slide. The slide was placed in a petri dish with filter papers moistened with sea water to prevent evaporation. In experiments where eggs were first pulsed with puromycin and then treated with ME, 1 ml of 1×10^{-3} M puromycin was mixed with 1 ml of fertilized egg suspension, washed three times after a 15 minute period of incubation and then subsequently, at different times, exposed to ME for 20 minutes.

Androgenic merogones were obtained by inseminating the enculeate fragments of eggs obtained by cutting (for detailed method see Rustad, Yuyama and Rustad, 1970). Briefly, unfertilized eggs were cut with a thin glass needle into approximately equal halves in a Syracuse dish coated with 0.2% agar. Enucleate and nucleate halves were sorted immediately after each cut, since the half containing a female pronucleus can be determined during the time of cutting. Approximately 30 minutes were required to cut 60 eggs. Extreme caution was taken to prevent polyspermy in fertilizing the cut eggs.

Determination of the incorporation of ^{3}H-leucine was performed in the following manner. Sea urchin eggs were washed twice with sea water filtered through Millipore filters, inseminated, washed and transferred into 10 ml of sea water containing either ME or puromycin. After 5 minutes, 1 μc/ml ^{3}H-leucine (5.0 c/mmole, New England Nuclear) was added. The reaction was terminated by adding an equal volume of 10% trichloroacetic acid (TCA) and the samples

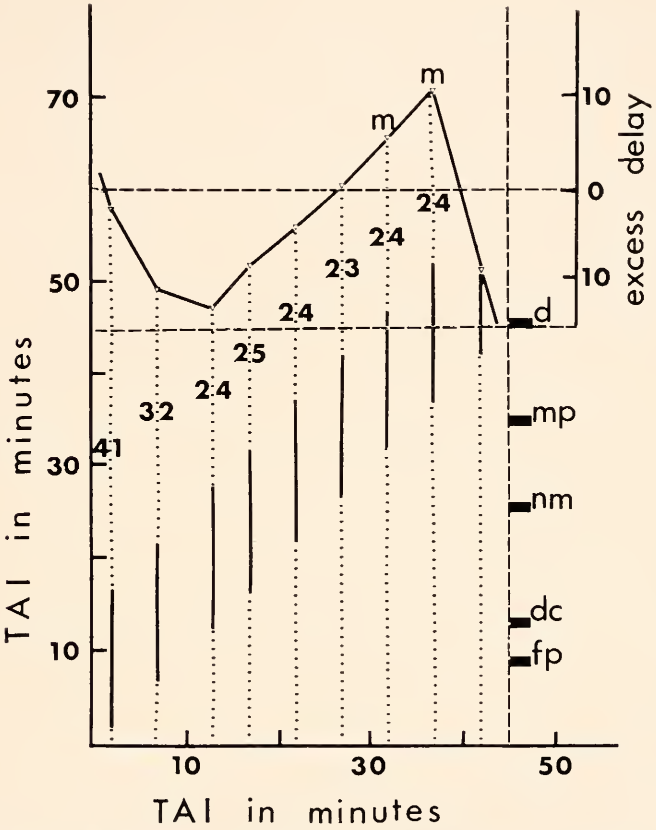

FIGURE 1. Delay-ME exposure diagram of *Lytechinus* eggs. Eggs were fertilized, and treated by ME for 15 minutes, initiated at different times after insemination. Lengths of each vertical line indicate the first cleavage period of *Lytechinus* eggs. Duration of the ME treatment is indicated by the solid line. Numbers associated with each vertical line indicate the recovery period. The mitotic stages of the control eggs are indicated on the right side of the graph. Abbreviations used are: TAI = time after insemination; m = multiple division in some eggs; d = time of control cleavage; mp = metaphase; nm = disappearance of the nuclear membrane; dc = centriole separation; fp = fusion of pronuclei; excess delay = cleavage period of ME treated eggs — cleavage period of control egg — duration of the ME treatment.

were heated for 15 minutes at 90° C. An excessive amount of non-radioactive leucine was added and the samples were kept overnight in the cold room. The samples were filtered through glass fiber filters, washed with 70% ethanol, dried, placed in vials containing toluene and proper fluors and the radioactivity was counted using a Nuclear Chicago Scintillation Counter.

RESULTS

Cleavage delay

In the first series of experiments, *Lytechinus* eggs were inseminated and exposed to 0.1 *M* ME for periods of 15 minutes, starting at various times after fertilization. Typical results are shown in Figure 1. During the first cleavage cycle, there was a change in delay, although the exposure period was kept constant. With batches of cells whose first division cycle was 45 minutes, the delay decreased when the treatments were initiated up until 13 minutes after insemination, increased linearly up until 37 minutes and decreased rapidly after this time. During the period when delay increased linearly (the linear delay phase), the interval between the end of exposure and the time of cleavage was constant (Fig. 1). Although the period of the first cleavage cycle varied among different

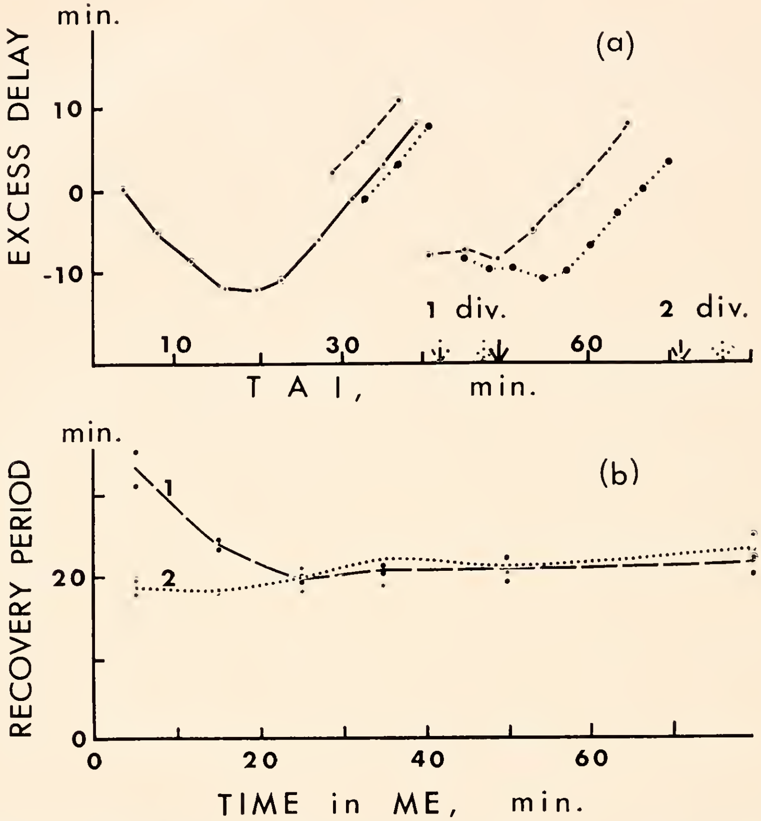

FIGURE 2. Delay-ME exposure diagram of *Arbacia* eggs. (a) The experimental design is similar to that of Figure 1, although eggs are treated for 20 minutes in this experiment. Different lines indicate separate experiments. (b) Dependence of the recovery period upon the duration of the ME treatment, the treatment was initiated either at the beginning of the streak stage (line 1) or several minutes before metaphase (line 2).

batches of eggs (45–60 minutes) and although the amount of delay varied with different experiments, the essential pattern of delay was the same in six different experiments. The start of the linear delay phase superficially corresponds to the beginning of the streak stage; division (separation) of the mitotic centers appears to occur about this time (Wilson and Mathews, 1895). The end of the linear delay phase corresponds approximately to metaphase after which sensitivity of cells to ME is rapidly lost. The above experiments established that the delay induced by ME treatment is not constant but fluctuates during the cleavage cycle.

In order to examine whether or not this fluctuation of delay would occur in another genus of sea urchin eggs, the same short exposure experiments were performed with *Arbacia* eggs. The delay pattern of *Arbacia* eggs was essentially the same as that of *Lytechinus* (Fig. 2a). In addition, the linear delay phase was also observed in the second division cycle. Since the linear delay phase reflects the constant period between the end of exposure and th time of cleavage (see Fig 1), it suggests that ME damages a certain event related to cytokinesis and that recovery from this damage takes a constant period of time. In order to examine whether this ME sensitive event is present from the beginning of the streak stage or develops at later stages, the dependence of the recovery period upon the exposure time to ME was studied. Fertilized eggs were exposed to ME for various periods of time starting at two different stages (beginning of the streak stage and immediately before the end of the linear delay phase; *ca.* metaphase). Typical results are shown in Figure 2b. When the ME treatments were initiated at the beginning of the streak stage, the recovery period gradually decreased and then became constant after periods of exposure for about 20 minutes or longer. When the exposure was initiated immediately before the end of the linear delay phase, the recovery period did not depend upon the period of exposure between 5 and 80 minutes.

These observations suggest that the ME sensitive event develops about 20 minutes after the beginning of the streak stage. This time is several minutes before metaphase. At about metaphase, eggs lose sensitivity to the ME treatment. In all experiments the recovery period (an interval between the end of exposure to ME and the time of cleavage) is 20–25 minutes and is longer than the interval between metaphase and cleavage (8–12 minutes).

In the next series of experiments, the effects of temperature on the recovery period were investigated. An Arrenius plot of the data indicated that the energy of activation for the recovery period is 16,400 calories.

In the third series of experiments, the relationship between protein synthesis required for division and the blockage of cleavage by ME was investigated in *Arbacia* eggs. Eggs were exposed to puromycin (5×10^{-4} *M*) immediately after fertilization for a period of 15 minutes. This treatment resulted in approximately 30 minute delays in cleavage; the synchrony of cleavage was not appreciably changed by the treatment. When these puromycin-treated cells were exposed briefly to ME at various times during the cycle, the appearance of the linear delay phase was delayed for approximately 30 minutes (Figure 3). In four separate experiments, there was a precise correlation between the delay of cleavage due to the puromycin treatment and the delay of the appearance of the linear delay phase. These results indicate that the ME sensitive event arises only after the protein synthesis required for division.

Arbacia eggs were continuously treated with puromycin. When the treatments were initiated at different times after fertilization, cleavage was blocked up until about 10 minutes after insemination. After this period, cleavage was delayed up until the beginning of the streak stage (delay decreased rapidly during this period) and then the first cleavage was no longer affected by the puromycin treatment. These findings confirmed the observation of Rustad and Burchill (1966). When the cells were treated with ME for 20 minutes during the puromycin sensitive period and then incubated immediately with puromycin ($5 \times 10^{-4}\,M$), it was found that sensitivity to puromycin is markedly reduced after the ME treatment. This suggests that protein synthesis required for division continues during the ME treatment. In order to verify this point, eggs were incubated with ^{3}H-leucine (1 μc/ml) for a period of 30 minutes in the presence of ME ($0.1\,M$) or puromycin ($5 \times 10^{-4}\,M$). The hot TCA insoluble radioactivity was determined. The average counts per minute (from two separate experiments) were:

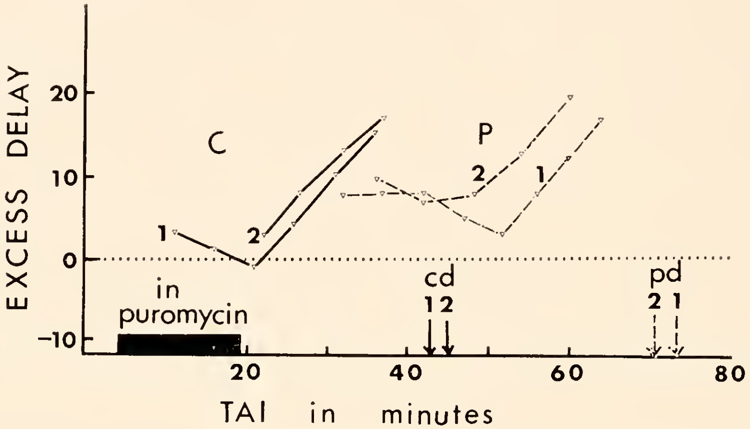

FIGURE 3. Delay-ME exposure diagram of the puromycin treated cells. The delay patterns of *Arbacia* eggs treated by ME alone (C) are compared with those pulsed with puromycin for 15 minutes before the ME treatment (P). Lines with the corresponding numbers represent experiments performed with the same batches of eggs. Abbreviations used are: cd = cleavage time of controls; cp = cleavage time of puromycin treated controls.

Control 8495; ME treated 6714; puromycin treated 1317.

During the linear delay phase, no additional delay was observed with eggs which were treated with puromycin after the ME treatment, suggesting that recovery from the ME treatment does not require protein synthesis.

Went (1962) reported that dithiodiglycol (DTDG), an oxidized product of ME, solated the mitotic apparatus and inhibited cleavage. Since the effective concentration of DTDG ($1 \times 10^{-3}\,M$) was much lower than that of ME ($1 \times 10^{-1}\,M$), there was a possibility that the effects of ME were due to DTDG contamination in the ME solution, as contamination by 1% DTDG in ME may produce the effect. The effects of DTDG ($1 \times 10^{-3}\,M$) on the cleavage of *Arbacia* eggs were studied. With DTDG the sharp transition of sensitivity which was the characteristic of the ME effect, was not observed during the first cleavage cycle. Eggs exposed to DTDG at later stage of the cycle showed a characteristic abnormality of furrow formation (wrinkled all over the egg surface). After short exposures to DTDG (20 or 30 minutes), eggs divided so asynchronously that it was impossible to estimate the 50% division time precisely. These observations rule out the possibility that the effects of ME observed in the present study were due to contamination of the ME solution.

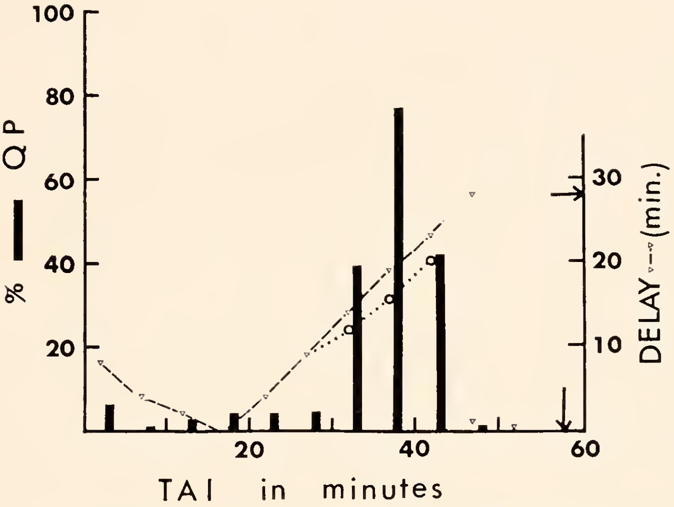

FIGURE 4. Early quadripartition in *Lytechinus* eggs. Experimental design is similar to that of Figure 1, although eggs were treated by ME for 20 minutes. Triangles = cleavage time of bipartitioned cells; circles = cleavage time of quadripartitioned eggs. Solid bars indicate the percentage of QP; arrows = cleavage time of controls.

Quadripartition

Frequencies of quadripartition (QP) varied widely in different experiments. Typically, a majority of cells underwent QP only after prolonged exposure to ME. Sometimes, however, high frequencies of QP were observed in *Lytechinus* eggs after a 15–20 minute exposure to ME. The result of one of these observations is shown in Figure 4. In this experiment, fertilized eggs were exposed to ME for a period of 20 minutes starting at different times after insemination. With a 58 minute cell cycle, frequencies of QP increased markedly (from 4% to 39%), starting at 32 minutes after insemination, reached a peak (77%) at 37 minutes and decreased (42%) at 42 minutes. At 47 minutes, some cells were no longer blocked and no significant percentage of QP was observed. In the case where 39% showed QP, cells were removed from ME 6 minutes before the onset of the first cleavage (not a single cell in the control started to divide at this time), and they divided 18 minutes before the second cleavage. In the case where 77% showed QP, cells were removed 1 minute before the first cleavage and they divided

15 minutes before the second cleavage. This phenomenon will be referred to as "early quadripartition." These observations demonstrate that it is not necessary to treat cells for a prolonged period of time to induce quadripartition. The majority of cells undergoing QP divided about 5 minutes earlier than bipartitioned cells. The phenomenon of "early QP" was not observed in *Arbacia* eggs, however.

In the next series of experiments, the role of the female pronucleus on QP was investigated. Whole eggs, encleate halves and nucleate halves of *Arbacia* eggs were inseminated and treated with ME for 30 minutes starting 30 minutes after the insemination. QP occurred in androgenic merogones in two separate experiments, indicating that the female pronucleus and half of the cytoplasm are not required for QP (Fig. 5).

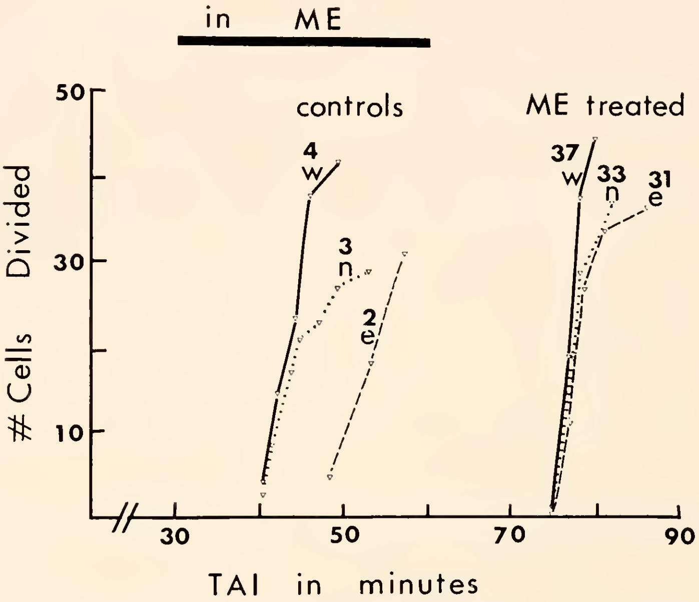

FIGURE 5. QP in the androgenic merogones of *Arbacia* eggs. Numbers associated with each line indicate the number of eggs undergoing QP; W = whole eggs; n = nucleated halves; e = enucleated halves.

The first cleavage of androgenic merogones obtained by cutting is always delayed for 5–15 minutes compared with diploid controls (Rustad *et al.,* 1970). It was found that this characteristic delay disappeared after the ME treatment (Fig. 5).

DISCUSSION

Cleavage delay

Delays induced by a short exposure to ME initiated at various times after fertilization depend upon the stage during the cleavage cycles. During the first and the second cycles, there exists a linear delay phase. This phenomenon occurs because the interval between the end of exposure to ME and the time of cleavage (recovery period) is constant. This indicates that ME damages a certain specific event required for cleavage and that recovery from this damage takes a constant period of time. The previous observation that the delay in cleavage is equal to the duration of exposure (Mazia, 1958) can be explained by assuming that the ME treatment was initiated at a certain stage such that the recovery period was equal to the interval between the time of the initiation of exposure and the time of control cleavage.

Although the existence of the linear delay phase can also be explained by an alternative possibility that ME merely arrests cleavage at a certain stage during the cycle, this interpretation is unlikely because of the following reasons. First, the recovery period (20–25 minutes in *Arbacia*) is much longer than the interval between the time when eggs become insensitive to ME and the time of cleavage (8–12 minutes). This longer duration of the recovery period cannot be explained by mere diffusion of ME from eggs, for the energy of activation of the recovery period is much greater than the diffusion process in general. In addition, it is known that the temperature coefficient of the processes between metaphase and cleavage is low (Ephrussi, 1933). Secondly, the effects of ME are not completely reversible; cells often divide into four after the ME treatment (Mazia and Zimmerman, 1958).

The results of studies investigating the dependence of the recovery period upon the duration of the ME treatment, indicated that the ME sensitive event develops several minutes before the transition point; the point at which eggs lose their sensitivity to ME. Thus the ME sensitive event must be present only briefly (for about 5 minutes) during the cleavage cycle. This duration is much shorter than the period during which MA is visible in the cell.

The furrowing itself is not blocked by ME, for the transition point occurs several minutes prior to the onset of the furrowing (Mazia, 1958). In addition, furrows induced by pressure centrifugation are not blocked by ME (Zimmerman, Murakami and Zimmerman, 1968). Thus, the mechanisms of the division block by ME must be sought in some events which occur prior to the furrowing.

Fluctuation of protein bound -SH groups in dividing sea urchin eggs appears to be linked to cytokinesis (Rapkin, 1931; Sakai and Dan, 1959). The fact that the pattern of delay induced by ME and by ether (Swann, 1954) are very similar and the fact that ether freezes the change of -SH groups in cortical proteins (Sakai, 1963) suggest that there is a close relationship between the activities of the -SH groups and the action of ME. However, if freezing of the changes in -SH groups is totally responsible for the blockage of division by ME, the recovery period should become progressively shorter. The results of the present experiments contradict this conclusion.

Puromycin inhibits protein synthesis and arrests cleavage at the streak stage in sea urchin eggs (Hultin, 1961). Cycloheximide also blocks cleavage in echinoderm eggs (Young *et al.*, 1969). Puromycin sensitivity is rapidly lost at about the time of the streak stage and the sensitivity period to puromycin is extended in the irradiated eggs (Rustad and Burchill, 1966) in which the streak stage is prolonged (Henshaw, 1940). These observations suggest that the protein synthesis required for cleavage is completed at some time during the streak stage. This is well before the development of a postulated ME sensitive event. The results of the present experiments revealed that (a) short exposure to puromycin delayed cleavage and postponed the appearance of the linear delay phase for an equal length of time, (b) eggs were not sensitive to puromycin after the ME treatment during the linear delay phase, (c) the ME treatment reduced puromycin sensitivity before the onset of the linear delay phase and (d) the incorporation of radioactive leucine into hot TCA insoluble fractions was not appreciably reduced during ME treatment. These observations suggest that (a) protein synthesis required for division continues during the ME treatment, (b) the ME sensitive event develops only after the synthesis of proteins required for cleavage and (c) recovery from ME treatment does not require protein synthesis.

The importance of MA in determining the orientation and the position of the division furrow in various cells has been discussed by many authors (Kawamura, 1960; Scott, 1960, 1965; Rappaport, 1965; see also Mazia, 1961 for review), although the cortical region of eggs acquires a capacity of autonomous furrowing prior to cytokinesis (Swann and Mitchison, 1953; Hiramoto, 1956. The oriented structure of MA appears disrupted during the ME treatment, but it regains its normal structure rapidly after the eggs are returned to normal sea water. However, cytokinesis does not start immediately (Mazia and Zimmerman, 1958). During the ME treatment, the characteristic birefringence of MA persists (Yuyama, unpublished). At the electron microscope level, the characteristic microtubules of MA are present, although they are arrayed haphazardly (Harris, 1962).

These observations are consistent with the idea that ME blocks cleavage not by destroying the structure of MA, but by affecting the major function of MA—determination of the position of the cleavage furrow in the cortical region. It is conceivable that the hypothetical damage by ME of the original furrowing position is related to quadripartition (QP), since the damage might alter the spacial orientation of the mitotic spindle and distort the animal vegetal axis.

Quadripartition (QP)

Since the position of the furrow is directly related to the structure and the position of MA (Mazia, 1961), it is obvious that the formation of a tetrapolar MA is responsible for QP (Mazia and Zimmerman, 1968). In normally dividing cells, a mitotic center contains two centrioles (de Harven and Bernhard, 1956; Harris, 1961; Costello, 1961), and this structural duplicity is presumably the basis for the formation of four functional poles in QP (Mazia *et al.*, 1960; Went, 1966). The present study has shown that *Lytechinus* eggs which were briefly treated with ME before the first division underwent QP well before the second division of controls. Although this observation is consistent with most of the important concepts developed by Mazia and his coworkers (Mazia *et al.*, 1960; Went, 1966), it appears to demand a slight modification of the hypothesis on QP, since the establishment of four poles occurs well before the normal mitotic schedule. The simplest interpretation would be to assume that ME functionally dissolves connections between the two centrioles in each center so that each centriole becomes independent during the recovery. The independence of each center in the tetrapolar MA seems certain, since the distribution of chromosomes to each of four blastomeres is random (Bibring, 1962). Recent electron microscopic studies on normally dividing HeLa cells, however, suggested that only one of the two centrioles might participate in spindle tubule formation (Robbins and Jentzsch, 1969). It is conceivable that ME actively causes the premature "splitting" of centrioles, a putative event which permits the members of a pair of centrioles to separate and function independently (Mazia *et al.,* 1960), prior to their duplication.

The induction of QP in androgenic merogones demonstrated that the female pronucleus and half the cytoplasm are not required for QP. This is consistent with the concept that mitotic centers are brought in by sperm after normal fertilization (Boveri, 1900; Dirksen, 1961). Since many agents such as ether (Wilson, 1902; Swann, 1954), benzimidazole (Went, 1966), dinitrophenol, NaN_3 and detergents (Kuno, 1954; Kuno-Kojima, 1960) and butanol (Yuyama, unpublished) can induce multipolar division, the -SH radical of ME is probably not important in inducing QP. ME (and hence -SH) may be unique, however, in that it inhibits the duplication of mitotic centers (Mazia *et al.,* 1960).

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SUMMARY

Eggs of *Arbacia punctulata* and *Lytechinus variegatus* were treated with 0.1 *M* 2-mercaptoethanol (ME) for short periods initiated at different times after fertilization. In both the first and second cleavage cycles, there was a phase during which cleavage delay increased linearly. This linear delay phase reflects the constant period of recovery from damage caused by ME treatment. The ME sensitive event is estimated to be present only briefly (about 5 minutes) immediately prior to the metaphase and appears to be associated with processes that determine the cleavage furrow. This ME sensitive event must develop only after completion of the puromycin sensitive event (protein synthesis required for division), because there is an exact correlation between the puromycin induced delay and delay in the appearance of the linear delay phase.

Lytechinus eggs treated briefly with ME before the first cleavage underwent quadripartition well before the second cleavage of controls. ME, therefore, appears to cause a premature splitting of centrioles prior to their duplication. Quadripartition can also be induced in androgenic merogones, verifying the old concept concerning the role of sperm centrioles in normal cleavage.

LITERATURE CITED

BIBRING, T., 1962. An analysis of experimentally induced four-cell division in developing echinoderm eggs. *Ph.D. thesis, University of Calif.,* Berkeley, California.

BOVERI, T., 1900. Zellen-Studien IV. Über die Natur des Centrosomen. *Jena. Z. Naturw.,* **35**: 1–220.

BUCHER, N. L. R., AND D. MAZIA, 1960. Deoxyribonucleic acid synthesis in dividing eggs of the sea urchin, *Strongylocentrotus purpuratus*. *J. Biophys. Biochem. Cytol.*, **7**: 651–655.

COSTELLO, D. P., 1961. On the orientation of centrioles in dividing cells, and its significance: a new contribution to spindle mechanises. *Biol. Bull.*, **120**: 285–312.

DE HARVEN, E., AND W. BERNHARD, 1956. Etude au microscope electronique de l'ultrastructure de centriole chez les vertébrés. *Z. Zellforsch.*, **45**: 378–398.

DIRKSEN, E. R., 1961. The presence of centrioles in artificially activated sea urchin eggs. *J. Biophys. Biochem. Cytol.*, **11**: 244–247.

EPHRUSSI, B., 1933. Contribution a l'analyse des premiers stades du developpement de l'oeuf. Action de la temperature. *Arch. Biol.*, **44**: 1–147.

FAUTREZ, J., AND N. FAUTREZ-FIRLEFYN, 1963. A propos de la localisation du noyau dans les blastomères de l'oeuf *d'Artemia salina*. L'influence du β-mercaptoethanol et due dithiodoglycol sur cette localisation. *Develop. Biol.*, **6**: 250–261.

FOTAKIS, N. S., 1963. Die Wirkung von β-Merkaptoäthanol und α-Piponsäure auf die HeLa-Zellkultur. *Exp. Cell Res.*, **30**: 600–602.

HARRIS, P. G., 1961. Electron microscope study of mitosis in sea urchin blastomeres. *J. Biophys. Biochem. Cytol.*, **11**: 419–431.

HARRIS, P. G., 1962. The effect of mercaptoethanol on the mitotic spindle. Volume II NN-1 in S. S. Breeze, Jr., Ed., *International Congress Electron Microscopy*. Academic Press, New York.

HARVEY, E. B., 1952. Electrical method of sexing *Arbacia* and obtaining small quantities of eggs. *Biol. Bull.*, **103**: 285.

HENSHAW, P. S., 1940. Further studies on the action of roentgen rays on the gametes of *Arbacia punctulata*. II. Modification of the mitotic time schedule in the eggs by exposure of the gametes to roentgen rays. *Amer. J. Roentgenol. Rad. Ther.*, **43**: 913–916.

HIRAMOTO, Y., 1956. Cell division without mitotic apparatus in sea urchin eggs. *Exp. Cell Res.*, **11**: 630–636.

HULTIN, T., 1961. The effect of puromycin on protein metabolism and cell division in fertilized sea urchin eggs. *Experientia*, **17**: 410–411.

KAWAMURA, K., 1960. Studies on cytokinesis in neuroblasts of the grasshopper, *Chortophaga viridifasciata* (de Geer). *Exp. Cell Res.*, **21**: 1–18.

KUNO, M., 1954. Comparative studies on experimental formation of multinucleated eggs of sea urchins by means of various agents. *Embryologia*, **2**: 43–49.

KUNO-KOJIMA, M., 1960. The effect of DNP and NaN_3 on fertilized eggs of the sea urchin with special reference to the induction of the abnormal cleavage. *Embryologia*, **5**: 71–77.

LIMBOSCH-ROLIN, S., 1963. Les effets du β-mercaptoéthanol et du dithiodiglycol sur la croissance de *Escherichia coli* et du *Saccharomyces cerevisiae*. *Exp. Cell Res.*, **29**: 61–72.

LIMBOSCH-ROLIN, S., AND J. BRACHET, 1961. Etude des effets du β-mercaptoéthanol et du dithiodiglycol sur la division cellulaire chez les oeufs d'Amphibiens. *Exp. Cell Res.*, **24**: 120–130.

MAZIA, D., 1958. SH compound in mitosis. I. The action of mercaptoethanol on the eggs of the sand dollar *Dendraster excentricus*. *Exp. Cell Res.*, **14**: 486–494.

MAZIA, D., 1961. Mitosis and the physiology of cell division. Pages 77–412 in J. Brachet and A. E. Mirsky, Eds., *The Cell, Vol. 3.* Academic Press, New York.

MAZIA, D., AND E. ZEUTHEN, 1966. Blockage and delay of cell division in synchronized population of *Tetrahymena* by mercaptoethanol (monothioethylene glycol). *C. R. Trav. Lab. Carlsberg*, **35**: 341–361.

MAZIA, D., AND A. M. ZIMMERMAN, 1958. SH compound in mitosis. II. The effect of β-mercaptoethanol on the structure of the mitotic apparatus in sea urchin eggs. *Exp. Cell Res.*, **15**: 138–153.

MAZIA, D., P. G. HARRIS AND T. BIBRING, 1960. The multiplicity of the mitotic centers and the time-course of their duplication and separation. *J. Biophys. Biochem. Cytol.*, **7**: 1–20.

MEEKER, G. L., 1964. The effect of β-mercaptoethanol on synchronously dividing populations of *Astasia longa*. *Exp. Cell Res.*, **35**: 1–8.

NATHANS, D., 1964. Puromycin inhibition of protein synthesis: Incorporation of puromycin into peptide chains. *Proc. Nat. Acad. Sci.*, **51**: 585–592.

PALMER, L., 1937. The shedding reaction in *Arbacia punctulata*. *Physiol. Zool.*, **10**: 352–367.

RAPKIN, L., 1931. Sur les processus chimiques au cours de la division cellulaire. *Ann. Physiol. Physicochim. Biol.*, **7**: 382–418.

RAPPAPORT, R., 1965. Geometrical relations of the cleavage stimulus in invertebrate eggs. *J. Theoret. Biol.*, **9**: 51–66.

ROBBINS, E., AND G. JENTZSCH, 1969. Ultrastructural changes in the mitotic apparatus at the metaphase-to-anaphase transition. *J. Cell Biol.*, **40**: 678–691.

RUSTAD, R. C., AND B. R. BURCHILL, 1966. Radiation-induced mitotic delay in sea urchin eggs treated with puromycin and actinomycin D. *Rad. Res.*, **29**: 203–210.

RUSTAD, R. C., S. YUYAMA AND L. C. RUSTAD, 1970. Nuclear cytoplasmic relations in the mitosis of sea urchin eggs. II. The division times of whole eggs, and haploid and diploid eggs. *Biol. Bull.*, **138**: 184–193.

SAKAI, H., 1963. Studies on sulfhydryl groups during cell division of sea urchin egg. VI. Behavior of -SH groups of cortices of eggs treated with ether-sea water. *Exp. Cell Res.*, **32**: 391–393.

SAKAI, H. AND K. DAN, 1959. Studies on sulfhydryl groups during cell division of sea urchin egg. I. Glutathione. *Exp. Cell Res.*, **16**: 24–41.

SCOTT, A. C., 1960. Furrowing in flattened sea urchin eggs. *Biol. Bull.*, **119**: 260–272.

SCOTT, A. C., 1965. A new concept of the mechanism of cytokinesis. *Biol. Bull.*, **127**: 389.

SWANN, M. M., 1954. The mechanism of cell division: experiments with ether on the sea urchin egg. *Exp. Cell Res.*, **7**: 505–517.

SWANN, M. M., AND J. M. MITCHISON, 1953. Cleavage of sea urchin eggs in colchicine. *J. Exp. Biol.*, **30**: 506–514.

WENT, H. A., 1962. Structural modification of the mitotic apparatus during the early cleavages in sand dollar eggs. *Chromosoma*, **13**: 219–242.

WENT, H. A., 1966. An indirect method to assay for mitotic centers in sand dollar (*Denraster excentricus*) eggs. *J. Cell Biol.*, **30**: 555–562.

WILSON, E. B., 1902. Experimental studies in cytology. II. Some phenomena of fertilization and cell division in etherized eggs. III. The effect on cleavage of artificial obliteration of the first cleavage furrow. *Arch. Entwicklungmech. Organismen Wilhelm Roux* **13**: 353–395.

WILSON, E. B., AND A. P. MATHEWS, 1895. Maturation, fertilization and polarity in the echinoderm egg. New light on the "quadrille of the centers." *J. Morphol.*, **10**: 319–342.

YOUNG, C. W., F. J. HENDLER AND D. A. KARNOFSKY, 1969. Synthesis of protein for DNA replication and cleavage events in the sand dollar embryo. *Exp. Cell Res.*, **58**: 15–26.

ZIMMERMAN, A. M., 1964. Effects of mercaptoethanol on the furrowing capacity of *Arbacia* eggs. *Biol. Bull.*, **127**: 345–352.

ZIMMERMAN, S. B., T. H. MURAKAMI AND A. M. ZIMMERMAN, 1968. The effects of selected chemical agents on furrow induction in the eggs of *Arbacia punctulata*. *Biol. Bull.*, **134**: 356–366.

ZOTIN, A. I., L. S. MILMAN AND V. S. FAUSTOV, 1965. ATP level and cleavage of sea urchin eggs *Strongylocentrotus drobachiensis* (O. F. Muller). *Exp. Cell Res.*, **39**: 567–576.

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A SUMMARY OF INVERTEBRATE LEUCOCYTE MORPHOLOGY WITH EMPHASIS ON BLOOD ELEMENTS OF THE MANILA CLAM, *TAPES SEMIDECUSSATA*[1]

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Metchnikoff (1968, page 76) in 1891 defined leucocytes as ". . . colorless cells, possessing one or rarely two nuclei and a protoplasmic body capable of amoeboid movement. In many invertebrates there is only one variety of mobile blood corpuscles containing a few sparse granules, whereas in certain others, such as many insects and molluscs, two varieties occur—granular leucocytes, with a large number of coarse granules, and hyaline leucocytes, with few or no granules."

Within the broad range of this definition, the literature has been abundantly supplied with an array of names assigned to leucocytic cells. Up to 76 different morphological varieties of cell types were classified from lamellibranch molluscs in one paper (Betances, 1922). A few general reviews are available for information in invertebrate hematology. Of some use are Andrew (1965), Hyman (1967) and other volumes in the same series; Grassé (1960) and other volumes in this series; Maupin (1969); and Cuénot (1891, 1897).

In this study, the morphology and morphometrics of the leucocytes of *Tapes semidecussata* are described and related to criteria for general leucocyte forms. The literature describing leucocyte form and function within the invertebrate phyla is summarized in Table I. This table is not a complete review of the known literature but is an attempt to reduce the number of named forms to broad categories of similar cytology and function.

METHODS AND MATERIALS

The Manila clam (Japanese littleneck, Philippine clam), *Tapes semidecussata* (*Protothaca* (*Venerupis*) *japonica* Deshayes; *Paphia philippinarus* Adams and

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TABLE I

Invertebrate leucocytes from Porifera to Chordata

Phylum and Class	General cell type	Published names and synonyms	Where observed	Morphology	Size	Properties and special characteristics
Porifera (1, 35)	Hyaline leucocyte	Scleroblast (1)	Mesoglea			Secrete spicules
		Archeocyte (35)	Mesoglea	Large nuclei, slight cytoplasm		Stem cell, phagocytosis and encapsulation
		Amoebocyte (1)	Mesoglea	Variable, cytoplasm with or without granules		Phagocytosis, nutritive and defense
Coelenterata (1)	Hyaline leucocyte	Interstitial cell (1)	Mesoglea	= Archeocyte		Stem cell, give rise to cnidoblasts, *etc.* Originate from endo- and exodermal cells.
Platyhelminthes (1, 36)	Leucocyte	Neoblast	Mesoderm			Stem cell or intermediate dedifferentiated cell with totipotency
Aschelminthes Rotifera (1)	Hyaline leucocyte	Ameboid cells (1)	Pseudocoel	Branched pseudopodia		Phagocytic, excretory?
Nemertea (1)	Hyaline leucocyte	Lymphocyte	Mesenchyme	Similar to those in circulatory system		Regeneration, gonad formation
		Hyaline amoebocyte	Circ. system			
	Granulocyte	Granular amoebocyte	Circ. system			
	Erythrocyte	"erythrocyte"	Circ. system	Flattened, round or oval		Oxygen transport
Mollusca Polyplacophora (1, 2, 3)	Hyaline leucocyte	Leucocyte (3)	Hemolymph	Round or oval cells, weakly basophilic cytoplasm	4–8 μ	Seasonal variations in formed elements noted
	Leucoblast	Leucoblast	Hemolymph	Smaller with strongly basophilic cytoplasm		
Monoplacophora (3)	Granulocyte	(3)	Hemolymph	Round or slightly oval cells with a few slightly basophilic granules in the cytoplasm	7–10 μ	*Neoplina galatheae*
Lamellibranchia (1, 2, 4, 5, 6, 7, 8, 9)	Hyaline leucocyte	Macronucleocyte	Hemolymph (smears)	Hyaline cytoplasm = vert. lymphocyte. Nuc. $\frac{1}{2}$ to $\frac{2}{3}$ dia. of cell. Chromatin clumped	10–20 μ dia.	7.5% of total FW mussels
	Acidophil	Granular eosinophilic amoebocyte	Hemolymph (smears)	Nuc. $\frac{1}{3}$ size of cell, eccentric & ovoid; nucleolus often visible. Chromatin lightly clumped	8–20 μ dia.	85% of total FW mussels
	Basophil	Granular basophilic amoebocyte (7)	Hemolymph (smears)	Nuc. $\frac{1}{3}$ to $\frac{1}{2}$ size of cell. Chromatin heavily clumped	7–12 μ dia.	7% of total FW mussels
	Granulocyte	Granulocyte (4)	Hemolymph	= Crustacean forms		*Cardium, Pecten maximus*
	Leucocyte	Lymphocyte (4)	Hemolymph	= Mammalian forms		*Cardium, Pecten maximus*
	Granulocyte	Coarse grained amoebocyte (5)	Hemolymph (smears & sections)	Birefrigent granules, colored or colorless, depending on species	8×12 μ	Precursor to two below?
	Granulocyte	Fine grained amoebocyte (5)	Hemolymph (smears & sections)	Birefrigent granules, colored or colorless, depending on species	8×12 μ	

TABLE I—*(Continued)*

Phylum and Class	General cell type	Published names and synonyms	Where observed	Morphology	Size	Properties and special characteristics
	Leucoblast?	Basophilic (5) amoebocyte (5)	Hemolymph (smears & sections	Narrow band of cytoplasm, no granules	8×12 μ	
	Erythrocyte	"erythrocyte"	Hemolymph	Variable shape	20×30 μ	Oxygen transport? Numerous in *Arca*.
	Acidophil	Coarsely granular amoebocyte (6)	Hemolymph (smears)	Large amt. cyto. granular eosinophila not in cytoplasm. Round to oval central nucleus		Largest form. *Cardium norvegicum.* 48% of tot.
	Acidophil	Finely granular eosinophil (6)	Hemolymph (smears)	Coarse eosinophilic granules, well defined		Slightly smaller. 44% of total
	Basophil	Basophilic corpuscle (6)	Hemolymph	Round nucleus and small amount of cytoplasm, basophilic		Smallest. 8% of total
	Granulocyte "Brown cell"	Brown cell (8)	Auricular pericardial gland	Applique nuc. (2.6×4.4 μ). Small amt. of clear cyto. Large vacuole (s) with brown pigment. No nucleolus	11×18 μ	Cell prob. related to or arise from leucocytes. Function in excretion, protection, defense?
	Granulocyte	Granular amoebocyte (9)	Hemolymph	Granules, yellow or yellowish-green. Ovoid nuc. Neutro-baso-philic cytoplasm fixed	9–13 nuc. 3×4 μ	Modified amoebocyte. Amoeboid but slow
	Leucocyte	Hyaline amoebocyte (9)	Hemolymph	= to above when fixed and stained. Ovoid nucleus	5–15 μ nuc. 5 μ	Function in phagocytosis and pinocytosis
Gastropoda (1, 2, 10, 11, 12, 14)	Leucocyte	Hyaline amoebocyte (2)	Hemolymph (smears)	Small, might enlarge to one filled with eosinophilous granules		*Viviparus*
	Leuocoyte	Lymphoid cells (10)	Hemolymph) (smears)	Usu. round, sometimes spindle-shaped. Thin margin of hyaline cytoplasm	3–7 μ	Living cells exhibit amoeboid activity with filiform and lobose pseudopodia. *Busycon*
	Granulocyte	Granular macrophage (10)	Hemolymph (smears)	Many neutrophilic granules. Nuc. w/o nucleoli, oval, bean-shaped or bilobed	10–12 μ	Most numerous, Moderately thigmotatic and filamentous, petaloid and lobose pseudopodia
	Acidophil	Eosinophilic granular amoebocyte	Hemolymph (smears)	Cytoplasm filled with well-defined granules. Nuc. usu. eccentric, round to oval	10 μ	Cell fragments were often noted as vesicles. 1 to 2% of total
	Leucocyte	"normal cells" (11)	Hemolymph (sections and smears)	Round cells with one or more "flat" pseudopodia	10–15 μ	*Lymnaea stagnalis*
		Leucocyte (11)	Hemolymph (sections and smears)	Are a phase of normal cells		*Lymnaea stagnalis*
		"small cells" (11)	Hemolymph (sections and smears)	W/o pseudopodia. May be an artifact and arise by breakup of normal cells		*Lymnaea stagnalis*

TABLE I—(*Continued*)

Phylum and Class	General cell type	Published names and synonyms	Where observed	Morphology	Size	Properties and special characteristics
	Granulocyte	Wandering cells (11)	Hemolymph	Large and granulated-possibly phagocytic "normal cells"		*Lymnaea stagnalis*
		Small rounded amoebocytes (11)	Hemolymph	Motile and contain granules		*Lymnaea stagnalis*
	Leucocyte and granulocyte	Amoebocyte (12)	In tissue	Considerable variation in size and form. Nuc. vesicular, round, oval or lentiform. Cyto. lightly basophilic with granules. Nuc. usu. eccentric with coarse chromatin	9 × 12 μ	No differential count. Very little activity was correlated with digestion and shell repair. Main activity related to defense
	Small leucocyte	Small amoebocyte Type A (14)	All tissues	Hyaline cytoplasm, slight margin around darkly stained nuclei	10–20 μ dia nuc.	Often large numbers are concentrated *Helix aspersa*
	Large leucocyte	Large amoebocyte Type B (14)	Esp. mantle tissue	Abundant cytoplasm—otherwise similar to above	10–50 μ	Actually thought to be two types, one from CT, other from mantle epithelia. *Helix aspersa*
Scaphopoda (13)	Leucocyte	Spherical leucocyte (13)	Hemolymph	Large nucleus surrounded by a strongly basophilic cytoplasm	12 μ dia	
	Leucocyte	Leucocyte (13)	Hemolymph	Spherical or kidney-shaped nuc. Weakly basophilic cytoplasm	8 μ dia	
	Leucocyte	Amoebocyte (13)	Hemolymph	Feebly basophilic cytoplasm	10–15 μ dia	
Annelida (1, 15, 16)	Basophil	Basophilic amoebocyte (15)	Coelomic fluid and sections	Vary greatly in size and shape. Rounded or oval with or w/o pseudopodia. Many with coarse basophilic granules. Small cells strain most deeply. Nuc. small, rounded often eccentric	10–5 μ dia	*Lumbricus*. All stages from lymphocyte-like to large hyaline forms. Note: large basophilic forms with acidophilic granules seen.
	Acidophil	Small acidophilic cell (15)	Coelomic fluid and sections	Usu. oval or round with pseudopodia. Nuc. well defined, central or eccentric with or w/o granules, which are usu. small	10–15 μ dia	Cell is unstable in certain media and granules may fuse
	Acidophil	Medium acidophilic cell (15)	Coelomic fluid and sections	Vary greatly in shape. Eccentric nuc. Fine and/or coarse granules. Latter may be basophilic	15–25 μ dia	
	Acidophil	Large acidophilic cell (15)	Coelomic fluid and sections	Oval to fibroblast-like shape. Fine granules usu. present often numerous. Basophilic granules or brown pigment may be seen	up to 50 μ dia	

TABLE I—(*Continued*)

Phylum and Class	General cell type	Published names and synonyms	Where observed	Morphology	Size	Properties and special characteristics
	?	Chloragogen cell (15)	Esp. in sections around gut	Packed with large rounded granules with an affinity for basophilic dyes. Nuc. small. Nucleolus present	50 μ dia	Granules appear to be lipoproteins. Cells are often phagocytized by leucocytes
	Leucocyte	Lymphocyte (16)	Coelomic fluid and sections	Cytology and stain response, resembles those of fish	4–5 μ	*Pheretima indica*. Described as a hemocytoblast. Phagocytic
	Leucocyte	Monocyte (16)	Coelomic fluid and sections	Cytology and stain response, resembles those of fish	7–10 μ	Phagocytic
	Granulocyte	Granulocyte (16)	Coelomic fluid and sections	Most numerous eosinophilic form	7–12 μ	
	Leucocyte	Lamprocyte (16)	Coelomic fluid and sections	Large leucocytes containing yellowish globular vacuoles	to 22 μ	Probably chloragogen cells
	Leucocyte	Linocyte (16)	Coelomic fluid and sections	Cytoplasm filled with single or multiple vacuoles	to 22 μ	
Sipunculida (17)	?	Urn cell	Coelomic fluid	Two separate cells. Anterior most is a vesicular cell, the base and neck of which fit into a saucer-shaped posterior ciliated glandular cell		Selectively scavenge foreign or morbid matter from the coelomic fluid
Arthropoda Insecta (18, 19, 20, 21, 22, 23, 33)	Leucoblast	Prohemocyte (19, 33)	Hemolymph	Small, pale grey, often intensely basophilic. Smallest of hemocytes	15 μ	Stem cells for plasmatocytes, granular hemocytes, cystocytes, spherule cells and adipohemocytes (33)
	Leucocyte	Plasmatocyte (19)	Hemolymph	Basophilic and pleomorphic. Many tend to send out thin ruffled cytoplasmic extensions	8 μ dia to 6×20 μ	Develop into vermiform cells (20). Important in CT formation (22)? Usu. most abundant cell type
	Granulocyte	Granular hemocyte (19)	Hemolymph	Have many distinct usu. round, colorless or acidophilic granules. Nuc. usu. centrally located	10–20 μ	In some species, turn into intense hyaline forms *in vitro* resembling cystocytes
	Granulocyte	Cystocyte Coagulocyte (19)	Hemolymph	Very unstable and rapidly turn into bright hyaline forms and disintegrate with a small, round cartwheel-like nucleus in a weakly basophilic cytoplasm		Prob. a specialized type of granular hemocyte. Induce coagulation
	Granulocyte	Spherule cells (19) Coarsely granular cells	Hemolymph	Round to oval with many large, usu. round, nonrefrigent, acidophilic, colorless or pale yellow inclusions	5–10 μ	As above. Break down to form intense hyaline forms. Important in trephocytosis, immunity (19)
	Granulocyte	Adipohemocyte (19) Spheroidocyte Lamprocyte (18, 20)	Hemolymph	Very large, refringent fat droplets and several other smaller, non-lipid granular inclusions present. Nucleus usu. eccentric	8–10 μ	Common in Lepidoptera. Similar to, but usu. smaller than body fat cells. Imp. in trephocytosis cells. (19). Prob. differentiated from plasmatocytes (18)
	Granulocyte	Crystal cell (23)	Hemolymph	Equivalent to the oenocytoids in morphological description		In *Drosophilia*

TABLE I—(*Continued*)

Phylum and Class	General cell type	Published names and synonyms	Where observed	Morphology	Size	Properties and special characteristics
	Leucocyte or granulocyte	Oenocytoid (18, 19) Large granular cell	Hemolymph	Usu. basophilic with either elaborate canaliculi or folded finely granular strands; or with distinct plate-like or rod-like crystals. 1 to 2 nuclei		Differentiated in live preps. Imp. in processes concerned in growth and molting (18), and trephocytosis (19)
	Leucocyte ?	Podocyte (19)	Hemolymph	Very large, flattened and plasmatocyte-like. 3–8+ long rigid cytoplasmic extensions	30 μ+ in each arm	Extended plasmatocytes often confused for podocytes (19), or transform into podocytes (21). Very rare (in 2 species)
	Leucocyte	Lamellocyte (23)	Hemolymph	A varient of the plasmatocyte with greater ability to encapsulate and greater cohesiveness		In *Drosophilia*
Crustacea (1, 5, 24, 25, 26, 27)	Hyaline leucocyte	Hyaline cells (25) Explosive corpuscle	Hemolymph	Nuc. deeply basophilic, central, 5–7 μ dia. Cyto. scant, light blue usu. w/o granules. Granules when present, very fine, light blue and in a perinuclear position	7–10 μ dia	Immature granulocyte (25), Many of living cells exhibit pseudopodia Crayfish
	Small granulocyte (neutrophil)	Small granulocyte (25)	Hemolymph	Nucleus central to eccentric. Granules vary from amphophilic to eosinophilic	9–18 μ dia	From Crayfish
	Large granulocyte (neutrophil)	Large granulocyte (25)	Hemolymph	Nucleus eccentric, 7–9 μ dia. Cyto. light-blue to colorless, densely packed with amphophilic to eosinophilic granules	18–35 μ dia	Most mature form (25). From Crayfish
	Hyaline leucocyte	Lymphoid cell (26) Hyaline (25)	Hemolymph	Round or spindle shaped. Cytoplasm slight, may contain a few granules. Round or indented nucleoate nucleus	5–10 μ	*Callinectes sapidus*. Similar to hemocytoblast (26). Some = to monocyte
		Semi-hyaline thigmotatic amoebocytic (26) Hyaline cell (25)		Shape as above. With few to many fine granules (mitochondria ?). Cytoplasm stains light blue	5–10 μ	Thigmatotic
	Acidophil Large granulocyte	Cells with coarse granules (26)	Hemolymph	Round to elongate, sometimes flattened shape. Granules vary between species, are round to spindle shaped up to 1.5 μ in dia	15–30 μ	Highly amoeboid
	Small granulocyte	Cells with medium granules (26)	Hemolymph	Granules usu. $\frac{1}{3}$ to $\frac{1}{2}$ dia of coarse type. Nuc., round to indented, usu. anucleolate. Shape variable	10–15 μ	In some species, the cells seem to disintegrate very rapidly: a possible clotting factor

TABLE 1—(*Continued*)

Phylum and Class	General cell type	Published names and synonyms	Where observed	Morphology	Size	Properties and special characteristics
	Hyaline leucocyte	Explosive corpuscle (24, 26, 27)	Hemolymph	=hyaline cells, cells with medium granules		Very labile cells (27)
Echinodermata Holothuroidea (1, 28, 30, 31)	Hyaline leucocyte	Lymphocyte (29)	Coelomic fluid	Large nucleus with fine chromatin surrounded by a thin margin of hyaline cyto. Nucleoli present. Larger cells usu. with cytoplasmic inclusions. Shape, spherical to oval	4–6 μ dia	Sometimes possess one to three filaform pseudopodia. Seen in all species
	Hyaline leucocyte	Phagocyte (29) Bladder amoebocyte	Coelomic fluid	Nucleus spherical to kidney-shaped. Cyto. with a clear ectoplasm and granular endoplasm	6–14 μ dia	Phagocytic and seen in all species. Possess one or more petaloid or bladder-like pseudopodia. Filaform pseudopodia formed when in contact with a flat surface
	Granulocyte	Colorless morula cell (29) Spherule cell (21)	Coelomic fluid	Spherical when at rest. Each contains a variable no. of colorless refractive spherules in a hyaline cyto. Nucleus when seen is eccentric and 2.5 μ dia	8–20 μ dia	Blunt pseudopodia are formed. Seen in all species
	Granulocyte (pigmented)	Colored morula cell (29) Spherule cell (31)	Coelomic fluid	=to above but the spherules are colored (color variable)		
	Leucocyte ?	Fusiform cell (29)	Coelomic fluid	Elongate cells tapering at either end. Have a refractile outer cytoplasm	6–12 μ long 1–3 μ dia	Seen in all species
	Erythrocyte	Hemocyte (29)	Coelomic fluid	Variety of forms and not uniform within an individual. Nuc. about 4 μ dia. Hemoglobin carried in the cytoplasm	8–11 μ	True red blood cells. Found in only a few species
	?	Crystal cells (29)	Coelomic fluid	Possess one (usu.) to 3 large rhomboidal crystals with a thin film of hyaline cyto. surrounding the crystal. A flattened nuc.	$10 \times 8 \times 5\ \mu$	
	?	Vibratile cells (29) Flagellated amoebocyte (31)	Coelomic fluid	Spherical cells with flagella-length 4 × dia of cell body. Cyto. with vacuoles, dark or colored granules. A small nucleus		
Echinoidea (1, 28–32, 35) (see 29, 30, and 34 for an extensive list)	Hyaline leucocyte	Phagocyte amoeboid form (32, 35)	Coelomic fluid	=Holothuroid form—lymphocyte	7–10 μ	Amoeboid movement increases with volume of cyto (32). Have "spiked" pseudopodia (28). Are phagocytic
	Hyaline leucocyte	Phagocyte Petaloid form (32)	Coelomic fluid	=Holothuroid form—phagocyte	7–10 μ	Prob. a morphological derivative of amoeboid forms (28). Phagocytic

TABLE I—(*Continued*)

Phylum and Class	General cell type	Published names and synonyms	Where observed	Morphology	Size	Properties and special characteristics
	Granulocyte	Colorless morula cells (29) White mulberry cells (28) Colorless trephocyte (32) Colorless spherule cell (34)	Coelomic fluid	=Holothuroiorm. Vary from spherical to ovoid	10–18 μ dia	Defined for *Arbacia* sp. where they account for 42% of all cellular elements in coelomic fluid (32)
	Granulocyte	Colored morula cell (29) Red mulberry cell (28) Eleocyte (29, 30) Colored or red trephocyte (32) Colored spherule cell (34)		=Holothuroid form. Green, yellow and red spherules have been seen. Spherules in red form sometimes smaller than in other forms	7×15 μ	For *Arbacia*. Red form (eleocyte) is rich in glycogen and contains echinochrome (28). CDC-Red 48% and green 10% (32)
	?	Large spherical cells (29, 30)	Coelomic fluid	Brown tinted cytoplasm filled with granules and vacuoles	9–26 μ dia	Found only in the irregular urchins so far
	Hyaline leucocyte ?	Explosive cell (29) Hyaline hemocyte (30) Pointed yellow cell ? (28)	Coelomic fluid	Pale yellow, hyaline and amoeboid cytoplasm. Numerous vacuoles in cytoplasm	9–14 μ	Found in all regular echinoids. Cells do not contain hemoglobin but do disintegrate rapidly upon contact with injured tissue (29)
	?	Vibratile cell (29, 31, 32, 34) "cellules vibratiles" (28)	Coelomic fluid	=Holothuroid form		Was shown to have a blade-like flagella. May be a precursor to phagocytes (32)
	?	Fusiform cell (28, 29)	Coelomic fluid	=Holothuroid form		
Asteroidea (1, 29, 30) (see 29 and 30 for an extensive listing)	Hyaline leucocyte	Bladder filaform phagocyte (29, 30)	Coelomic fluid	=Holothuroid form. Both types appear to be phases of one cell type		Abundant in coelomic fluid
	Granulocyte	Colorless morula cell (29, 30)		=Holothuroid form		Found in small numbers
Crinoidea (1, 5, 29, 30)	Granulocyte	Colored and colorless morula cell (5, 29)	Coelomic fluid	=Holothuroid form		
	Hyaline leucocyte	Phagocyte (29)	Coelomic fluid	Large nucleus and cytoplasm which often contains granules. Short pseudopodia and active phagocytes	11 μ dia	Cannot yet be correlated with other echinoderm leucocytes
	?	Cell with rods and granules	Coelomic fluid	Elongate and usu. vermiform. Granules and rods have an affinity for saffranin. Nucleus with no fixed position	5×30 μ	

TABLE I—(*Continued*)

Phylum and Class	General cell type	Published names and synonyms	Where observed	Morphology	Size	Properties and special characteristics
Ophiuroidea (1, 29, 30)	Hyaline leucocyte	Phagocyte (29)	Coelomic fluid	=Crinoid form		Common in *Gorgonocephalus*
	Hyaline leucocyte	Hemocyte (1, 29)	Ambulacral system only	Size variable, most are spherical	1.5–10.5 μ	Contain a heme pigment possibly hemoglobin
	Granulocyte	Colorless morula cell (29)	Coelomic fluid	=Holothuroid form		In *Ophiopholis aculeata*
		Vibratile cell (29)	Coelomic fluid	=Holothuroid form		In *Ophiopholis aculeata*
		Fusiform cell (29, 30)	Coelomic fluid	=Echinoid form		In *Gorgonocephalus*
Chordata Tunicata-Ascidia (1, 31)	Hyaline leucocyte	Amoeboid leucocyte (1, 31)	Coelomic fluid	Lymphocyte-like with some of the larger forms, highly phagocytic ("macrophages")		Morphological derivatives reticulated amoeboid cell, and various vacuolated cells (non-amoeboic) (1)
	Granulocyte (pigmented)	Mulberry cells (1, 31) Colored cell	Coelomic fluid	Contain granules—blue, orange, green or brown colors. Slow amoeboid movement		Green cells contain a vanadium pigment of unknown function

References in Table I

1. Andrew, W. (1965)
2. Hyman, L. H. (1967)
3. Arvy, L. and M. Gabe (1949)
4. Betances, L. M. (1922)
5. Cuénot, L. (1891)
6. Drew, G. H. and B. A. Cantab (1910)
7. Dundee, D. S. (1953)
8. Haiger, S. A. (1964)
9. Takatsuki, S. (1934)
10. George, W. C. and J. H. Ferguson (1950)
11. Müller, G. (1956)
12. Pan, C. (1958)
13. Arvy, L. and M. Gabe (1951)
14. Wagge, L. E. (1955)
15. Cameron, G. R. (1932)
16. Kindred, J. E. (1929)
17. Bang, F. B. and B. G. Bang (1962)
18. Wigglesworth, V. B. (1959)
19. Jones, J. C. (1962)
20. Jones, J. C. (1959)
21. Rizki, M. T. M. (1957)
22. Wigglesworth, V. B. (1956)
23. Rizki, M. T. M. (1962)
24. Haughton, I. (1934)
25. Wood, P. J. and J. P. Visentin (1967)
26. George, W. C. and J. Nicols (1948)
27. Florkin, M. (1960)
28. Abraham, M. (1963)
29. Endean, R. (1966)
30. Boolootian, R. A. (1962)
31. Andrew, W. (1962)
32. Liebman, E. (1950)
33. Jones, J. C. (1964)
34. Johnson, P. T. (1969)
35. Cheng, T. C., H. W. F. Yee, E. Rifkin and M. D. Kramer (1968)
36. Rose, C. and S. Shostak (1968)

Reeve) (Cahn, 1951) was collected from the grounds of the Western Oyster Co. in Burley Lagoon, a confined bay in southern Puget Sound near Purdy, Washington. The clams were held at the Northwest Marine Health Sciences Research Laboratory (Purdy, Washington) in large flats supplied with ultraviolet light-treated flowing sea water.

Animal preparation: fixation, cutting and staining

Animals were routinely fixed in cold Davidson's fixative (formalin-acetic acid-alcohol modified with sea water) and transferred to 50% and 70% ethanol. A few samples were fixed in gluteraldehyde-formalin in preparation for methacrylate embedding.

Blocks for paraffin embedding were taken from sagittal sections (anterior to posterior axis of the adductor muscles) or from mantle tissue, and carried through a graded series of alcohol, xylene and paraffin. Sections were cut on a rotary microtome at 5 to 7 μ (1 to 2 μ for plastic). The slides were then stained in Harris hematoxylin and eosin. Plastic embedded sections were stained with toluidine blue.

Fluid from the pericardial cavity was obtained before the clams were fixed, and the blood cells were observed in the unfixed state with phase-contrast optics or fixed, stained and observed with bright-field optics.

Fixed and stained preparations were obtained by allowing a sample (0.01 ml or greater) of blood to settle on a glass slide for approximately five minutes, fixing these cells for five minutes in 4% formalin in sea water and then rinsing the slide with 95% ethanol and allowing it to air dry. The prepared slides were stained directly after fixation with Giemsa stain diluted 1:20 with Sorensen's phosphate buffer at pH 7.5 and 3% absolute methanol. After staining for 20 minutes the slides were rinsed with pH 7.5 buffer and air dried.

Cell counts

Cell counts were conducted on five animals with a modified Neubauer counting chamber. The counts were made directly on living cells without dilution. Cell morphometrics were determined in living and fixed preparations using an eyepiece micrometer.

Differential analyses of leucocytes in connective tissue underlying midgut and style sac epithelia were conducted on clams held for 1 to 30 days and 30 to 50 days in the Purdy Laboratory. To maintain as much uniformity in the counts as possible, the leucocytes were classified, with respect to nuclear diameter, as follows: small, 1.5 to 3 μ; medium, 3 to 6 μ; and large, 6 to 8 μ. Nuclei less than or equal to 3 μ were placed in the small class while those greater than or equal to 6 μ were classed as large. Total cell size was estimated visually by the relationship of a medium nucleus to cytoplasmic radius. A cytoplasmic radius of less than one nuclear diameter designated a small cell, equal to one nuclear diameter a medium cell, and greater than one nuclear diameter, a large cell. Cells which exhibited heterochromatic DNA were differentiated in the counts from euchromatic forms. All differential cell counts were conducted with phase-contrast optics to facilitate recognition of cell boundaries.

RESULTS AND DISCUSSION

Distribution

The leucocytes of *T. semidecussata*, like those of vertebrates, are transient cells, found in varying numbers in all tissues, including the muscle and kidney. They

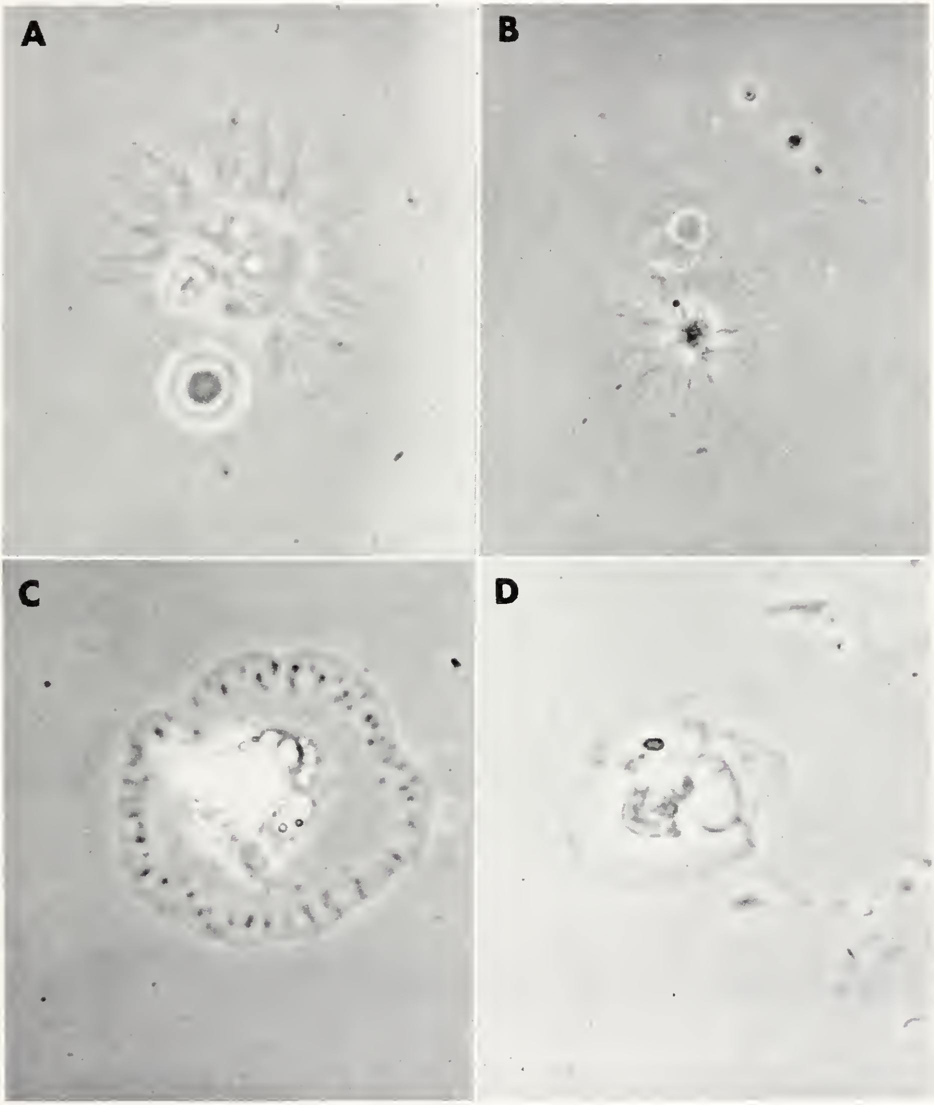

FIGURE 1. Living unstained leucocytes from *T. semidecussata*, A. Hyaline leucocyte with moderately filamentous pseudopodia, B. similar to A, but smaller and with greater radiation of pseudopodia, C. Hyaline leucocyte, with central granulation and peripheral cytoplasmic concentrations, D. similar to C, but without granulation and with petaloid pseudopodia, 1150 ×.

represent a distinct and identifiable group dispersed among the fibrous reticulum of loose connective tissue and between epithelial cells and muscle fibers, and are especially abundant in the subepithelial areas surrounding the GI tract, between gill lamellae and beneath the mantle epithelium. Normal appearing animals usually presented a pattern of diffusely dispersed leucocytes with some localized concentrations mainly beneath the mantle epithelium (small and medium forms) and gill lamellae connective tissue (large pigment cell forms). Hyaline and pigmented leucocytes were often infiltrated into the gastric epithelium, especially in the hindgut.

Yonge (1923) observed a similar distribution of leucocytes in the epithelium and connective tissues of the clam, *Mya arenaria*. In this regard, Haughton (1934), Wagge (1955) and Owen (1966) have reviewed literature referring to distributional patterns of leucocytes in other molluscs, especially gastropods.

Morphometrics

Many morphologically different leucocytes forms were observed both in tissue section and in fixed and living cells drawn from the pericardial cavity; however, most differences seemed to be correlated with the various preparative techniques employed. Paraffin-embedded material stained with hematoxylin and eosin, with observations mainly centered on connective tissue of the gill, mantle and GI tract, did not usually resolve the acidophilic granularity. Nuclear size, shape and position, and total cell size and shape remained as the principal gross features discriminating the cells.

The acidophilic cells in fixed suspensions generally had ovoid and eccentric nuclei, 1.4 by 2.9 microns, and the granules, when seen, were numerous. Preparations from some individuals revealed many heterochromatic nuclei in such cells. Nuclei of the hyaline leucocytes were usually round and central and varied greatly in size from 3 to 8 microns. Total cell size of these forms was extremely variable and ranged from 4 to 12 microns. At times, examples of the "pigment or brown" cells were observed in living cells, and some cells which approached the form of leucoblasts were seen. All cells seen in pericardial fluid formed pseudopodia (Figs. 1 and 2). Dissociated epithelial cells also exhibited pseudopodia formation and amoeboid movement. Granulocytic leucocytes may be distinguished in life by "spike" pseudopodia and very little motion. Hyaline leucocytes usually exhibit lobate pseudopodia and rapid motion. Both seem to be able to form branched filamentous pseudopodia (Figs. 1a, 2b).

Morphological features of the cell groups

The largest leucocytic cell observed in tissue section appeared similar to the vertebrate hemocytoblast (Bloom and Fawcett, 1962). It was infrequently seen in the Giemsa preparations and formed a homogeneous group in the maximum size range (6 to 8 μ). The next major size range, cells with medium-size nuclei (3 to 6 μ), was a heterogeneous population and represented the largest proportion of all leucocyte types found in tissue sections and in pericardial fluid. The smaller forms were the most abundant in tissue sections, but it must be noted that packing in sections does not always make them comparable with those seen in the pericardial fluid. Generally it may be concluded that the majority of medium leucocytes have a hyaline cytoplasm in which acidophilic granules can be resolved with oil immersion in $1\frac{1}{2}\ \mu$ plastic sections. Smaller cells appear to have fewer acidophilic granules, while the largest forms are more commonly amphoteric and basophilic. The nuclei of the medium-sized cells grade rather imperceptibly into the small nuclei groups (1.5–$3\ \mu$). Between medium-sized and small cells many transitional types

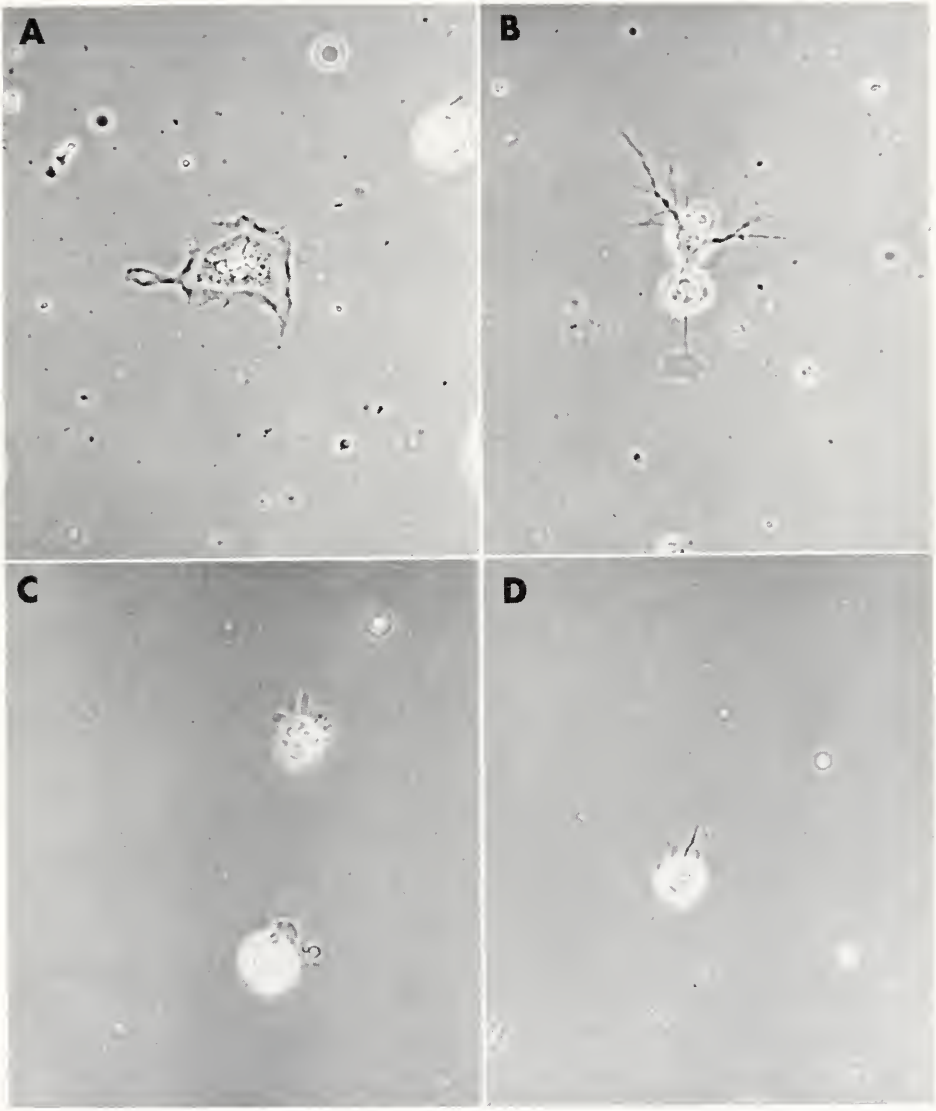

FIGURE 2. Living, unstained leucocytes from *T. semidecussata,* A. Hyaline leucocyte with lobate pseudopodia and an eccentric nucleus, B. Granulocyte with extensive filamentous pseudopodia, C. Granulocyte (lower) with hyaline cytoplasm occupying one pole, D. granulocyte with thin pseudopodia where adhered to glass; probably a morphological variant of C, 440 ×.

occur in contrast to the sharp differences noted between large and medium types. The cells with small nuclei are round or extremely elongate. Small elongate, irregular nuclei were usually euchromatic; however, heterochromatic or pycnotic round nuclei (usually 1.5–3 μ) were seen in varying proportions depending on the sample time and staining intensity. This heterochromasia was usually but not always associated with large cells (small nuclei) containing many granules. In bright field, with unstained preparations, this granularity presented a yellow to yellowish-brown coloration, the latter defined as a pigment cell character. The nuclei of all cells seemed to become more eccentric as granularity increased and the shape remained round except when compressed against the cell membrane. Except for some large leucocytes, nucleoli were not visible.

TABLE II

Differential counts of connective tissue leucocytes in the Manila clam, Tapes semidecussata. *Group I ($n = 14$) was held for 1 to 30 days in running seawater. Group II ($n = 14$) was held for 30 to 50 days in the same system. 100 cells were counted per individual*

Group	Nucleus dia (μ)	Cell dia (μ)	Mean %	S.D. ±%	Subgroup total %	Group	Nucleus dia (μ)	Cell dia (μ)	Mean %	S.D. ±%	Subgroup total %
I	1.5–3	3–6	11.3	5.9	40.2	II	1.5–3	3–6	14.0	7.1	59.0
		6–12	19.5	5.5				6–12	32.1	12.8	
		12–16	9.4	3.5				12–16	12.9	4.7	
	3–6	3–6	31.6	12.3	53.9		3–6	3–6	21.1	9.8	37.8
		6–12	18.4	6.2				6–12	12.9	7.3	
		12–16	3.9	2.2				12–16	3.8	2.9	
	6–8	3–6	1.2	1.5	5.9		6–8	3–6	1.0	1.3	3.2
		6–12	2.9	1.7				6–12	1.5	1.9	
		12–16	1.8	1.3				12–16	0.7	1.3	
	1.5–3*	All	24.5	6.6			1.5–3*	All	40.0	13.2	

* Heterochromatic nuclei.

Changes in the proportions of leucocytes possibly due to prolonged storage of clams under artificial conditions are shown in Table II. A comparison of differences in relative numbers between cell sizes and groups held for 1 to 30 days and 30 to 50 days suggest an increase in small-heterochromatic forms in the latter group. Tests employing the single classification analysis of variance were conducted to determine the significance of differences between groups for each nuclear size classification shown in Table II. The significance levels for acceptance of the hypothesis that the means of both groups were similar ($M_1 = M_2$) were as follows: all small forms, $P < 0.001$ ($F = 18.74$); heterochromatic small forms, $P < 0.001$ ($F = 19.68$); medium forms, $P < 0.005$ ($F = 10.57$); and large forms, not significant ($F = 2.31$). The heterochromasia exhibited by many small cells probably indicates some loss of cell function or degeneration (Robbins, 1967). The gross and histologic appearance of both groups was similar.

Summary

The morphology and known functions of leucocytes have been tabulated and compared for most invertebrate phyla. The leucocytes of the Manila clam are similar in form to molluscan leucocytes described by other investigators. Two general types were seen. One was a hyaline leucocyte ("leucocyte") and the other a granular leucocyte ("granulocyte"). Cells in these categories showed extreme variations in size and staining characteristics. A decrease in nuclear size often was correlated with an increase in nuclear heterochromasia and eccentricity and cytoplasmic granularity. The average cell count for all circulating leucocytes was 1650 ± 180 per mm^3.

LITERATURE CITED

Abraham, M., 1963. Étude morphologigae sur les coelomocytes des Echinides reguliers (A morphological study on the coelomocytes of regular echinoides). *Israel, J. Zool.*, **12**(1): 101–116.

Andrew, W., 1962. Cells of the blood and coelomic fluids of tunicates and echinoderms. *Amer. Zool.*, **2**(3): 285–297.

Andrew, W., 1965. *Comparative Hematology.* Grune and Stratton, New York, 188 pp.

Arvy, L., and M. Gabe, 1949. Contribution a l'étude morphologique du sang des Polyplacophores. *Bull. Soc. Zool. France*, **74**: 173–179.

Arvy, L., and M. Gabe, 1951. Donnees morphologiques sur le sang du Dentale. *Bull. Lab. Maritime Dinard.*, **35**: 15–22.

Bang, F. B., and B. G. Bang, 1962. Studies on sipunculid blood; immunologic properties of coelomic fluid and morphology of "urn cells." *Cah. Biol. Mar.*, **3**: 363–374.

Betances, L. M., 1922. Les cellules du sang de quelques Lamellibranches. *Arch. D'Anat. Microscop.*, **18**: 309–327.

Bloom, W., and D. W. Fawcett, 1962. *A Textbook of Histology.* Saunders, Philadelphia, 720 pp.

Boolootian, R. A., 1962. The perivisceral elements of echinoderm body fluids. *Amer. Zool.*, **2**: 275–284.

Cahn, A. R., 1951. Clam culture in Japan. *U. S. Fish. Wildl. Serv. Fish. Leafl.*, **399**: 1–80.

Cameron, G. R., 1932. Inflammation in earthworms. *J. Pathol. Bacteriol.*, **35**: 933–972.

Cheng, T. C., H. W. F. Yee, E. Rifkin and M. D. Kramer, 1968. Studies on the internal defense mechanisms of sponges, III. Cellular reactions in *Terpios zeteki* to implanted heterologous biological materials. *J. Invert. Pathol.*, **12**: 29–35.

Cuénot, L., 1891. Études sur les sang et les glandes lymphatiques dans la sèrie animale (2^{e} partie: Invertèbrés). *Arch. Zool. Exp. Gén. Sèrie 2*, **9**: 13–90, 365–475, 593–670.

Cuénot, L., 1897. Les globules sanguins et les organes lymphoides des invertèbrés. *Arch. Anat. Microscop.*, **1**: 153–192.

Drew, G. H., and B. A. Cantab, 1910. Some points in the physiology of lamellibranch blood corpuscles. *Quart. J. Microscop. Sci.*, **54**(4): 605–622.

Dundee, D. S., 1953. Formed elements of the blood of certain freshwater mussels. *Trans. Amer. Microscop. Soc.*, **72**(3): 254–264.

Endean, R., 1966. The coelomocytes and coelomic fluids. Pages 301–328 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience, New York.

Florkin, M., 1960. Blood chemistry. Pages 141–160 in T. H. Waterman, Ed., *The Physiology of Crustacea, Vol. I.* Academic Press, New York.

George, W. C., and J. H. Ferguson, 1950. The blood of gastropod molluscs. *J. Morphol.*, **86**: 315–327.

George, W. C., and J. Nichols, 1948. A study of the blood of some Crustacea. *J. Morphol.*, **83**(3): 425–443.

Grassé, P., Ed., 1960. *Traité de Zoologie, Vol. V.* Masson, Paris.

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Haiger, S. A., 1964. A histological and cytological study of the "brown cells" found in the "auricular pericardial gland" and other tissues of the oyster, *Crassostrea virginica* (Gmelin). *M.S. thesis, University of Delaware,* 51 pp.

Haughton, I., 1934. Amoebocytes and allied cells in Invertebrata. *J. Roy. Microscop. Soc.,* **54**: 246–262.

Hyman, L. H., 1967. *The Invertebrates, Vol. VI, Mollusca I.* McGraw-Hill, New York, 792 pp.

Johnson, P. T., 1969. The coelomic elements of sea urchins (*Strongylocentrotus*) I. The normal coelomocytes; their morphology and dynamics in hanging drops. *J. Invert. Pathol.,* **13**: 25–41.

Jones, J. C., 1959. A phase contrast study of the blood cells of *Prodenia* larvae. *Quart. J. Microscop. Sci.,* **100**(1): 17–24.

Jones, J. C., 1962. Current concepts concerning insect hemocytes. *Amer. Zool.,* **2**(2): 209–246.

Jones, J. C., 1964. The circulatory system of insects. Pages 2–107 in M. Rockstein, Ed., *The Physiology of Insects, Vol. III.* Academic Press, New York.

Kindred, J. E., 1929. The leucocytes and leucocytopoietic organs of an Oligochaete *Pheretima indica* (Horst). *J. Morphol.,* **47**(2): 435–467.

Liebman, E., 1950. The leucocytes of *Arbacia punctulata. Biol. Bull.,* **98**: 46–59.

Maupin, B., 1969. *International series of monographs in pure and applied biology. Division: Zoology. Vol. 41. Blood platelets in man and animals. Vol. 1 and 2.* Pergamon Press, New York.

Metchnikoff, E., 1968. *Lecture on the comparative pathology of inflammation, delivered at the Pasteur Institute in 1891.* Dover, New York.

Müller, G., 1956. Morphologie, Lebensablauf, and Bildungsort der Blutzellen von *Lymnaea stagnalis* L. (Course of life and site of formation of the blood cells of *L. stagnalis* L.). *Z. Zellforschung,* **44**: 519–556.

Owen, G., 1966. Feeding. Pages 1–52 in K. M. Wilbur and C. M. Yonge, Ed., *Physiology of Mollusca,* Vol. II. Academic Press, New York.

Pan, C., 1958. The general histology and topographic microanatomy of *Australorbis glabratus. Bull. Mus. Comp. Zool., Harvard Univ.,* **119**: 237–290.

Rizki, M. T. M., 1957. Alteration in the haemocyte population of *Drosophila melanogaster. J. Morphol.,* **100**(3): 437–458.

Rizki, M. T. M., 1962. Experimental analysis of hemocyte morphology in insects. *Amer. Zool.,* **2**(2): 247–256.

Robbins, S. L., 1967. *Pathology.* Saunders, Philadelphia, 1433 pp.

Rose, C., and S. Shostak, 1968. The transformation of gastrodermal cells to neoblasts in regeneration *Phagocata gracilis* (Leidy). *Exp. Cell. Res.,* **50**: 553–561.

Takatsuki, S., 1934. On the nature and function of the amebocytes of *Ostrea edulis. Quart. J. Microscop. Sci.,* **76**: 379–431.

Wagge, L. E., 1955. Amoebocytes. Pages 31–78 in G. H. Bourne, and J. F. Danielli, Eds., *International review of cytology,* Vol. IV. Academic Press, New York.

Wigglesworth, V. B., 1956. The haemocytes and connective tissue formation in the insect, *Rhodnius prolixus* (Hemiptera). *Quart. J. Microscop. Sci.,* **97**: 89–98.

Wood, P. J., and L. P. Visentin, 1967. Histological and histochemical observations of the hemolymph cells in the crayfish, *Orconectes virilis. J. Morphol.,* **132**(4): 559–567.

Yonge, C. M., 1923. The mechanism of feeding, digestion and assimilation in *Mya arenaria. J. Exp. Biol.,* **1**(1): 15–63.

Reference: *Biol. Bull.*, **140**: 369–375. (June, 1971)

REMOVAL OF THE FERTILIZATION MEMBRANE OF FERTILIZED EGGS OF *URECHIS CAUPO* AND DEVELOPMENT OF "MEMBRANELESS" EMBRYOS[1]

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Embryos of *Urechis caupo* are invested by a fertilization membrane, and unlike many other invertebrates, embryos of *Urechis* do not lose this membrane during "hatching" of the blastulae. The removal of the fertilization membrane is a prerequisite to embryo dissociation; embryo dissociation has been employed recently to investigate the role of cell interaction in the regulation of biosyntheses (Giudice, Mutulo and Moscona, 1967; Pfohl and Giudice, 1967; Shiokawa and Yamana, 1967; Sconzo, Pirrone, Mutolo and Giudice, 1970; Engstrom, in preparation). This paper describes a method to remove the fertilization membrane and the subsequent development of the "membraneless" embryos.

MATERIALS AND METHODS

Collection and culture of gametes

Specimens of *Urechis caupo* collected from the mudflats of Bodega Bay, California, were housed in aquaria containing aerated recirculating sea water at 8–12° C. The collection and filtering of eggs has been described (Gould, 1967). Unless otherwise stated, all incubations of eggs and embryos were at 15° C. A one per cent suspension of eggs (vol/vol) was inseminated with a fresh suspension of sperm. Five minutes later, those eggs destined for demembranization were washed and resuspended in millipore-filtered sea water (FSW). Control eggs (with membranes) were similarly washed, resuspended at 2° C in FSW containing 0.1 mg each of penicillin and streptomycin/ml (PSSW) and then transferred to a 15° C water bath for subsequent development. Embryos were cultured in silicone-treated glass petri dishes in volumes not exceeding 5 mm in height in order to allow good aeration and development of the embryos. Untreated glass, agar-coated glass and plastic were not suitable containers for culturing membraneless embryos due to adherence of denuded embryos to these substrates.

Removal of the fertilization membrane

When the fertilization membrane (FM) commenced to rise (10–15 minutes post fertilization), eggs were centrifuged, resuspended in calcium-free sea water (28.4 g NaCl, 5.4 g $MgCl_2 \cdot 6H_2O$, 0.78 g KCl, 7.12 g $MgSO_4 \cdot 7H_2O$, 0.2 g $NaHCO_3$ per liter, pH 7.8—all reagents were analytical grade from Mallinckrodt) containing 1 mM disodium ethylenediamine tetraacetate, pH 7.8–7.9 (CFSW-EDTA), centrifuged and finally resuspended at 2° C in 20–25 volumes of a sucrose-EDTA solution (SE)—0.87 M sucrose, 5 mM EDTA, pH 7.8–7.9. The

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egg suspension was transferred to a 15° C water bath and periodically inverted very gently to mix the suspension. Fertilization membranes became progressively thinner until they were no longer visible by light microscopy; this typically required 10–15 minutes. At the first sign of complete dissolution of the FM, the suspension was gently diluted with 30 volumes of FSW. The above treatments always resulted in the lysis of some eggs (5–20%). The final suspension was centrifuged at 40 × *g* for $\frac{1}{2}$ minute in a Sorvall GSA rotor. These demembranated eggs were resuspended at a concentration of 1% in PSSW (assuming no loss of eggs during SE treatment) and cultured under the same conditions as the controls. When necessary, the cultures were gently rotated to resuspend any embryos adhering to the siliconized glass.

Mechanical treatment, thiol reagents, proteolytic enzymes, nonelectrolytes and combinations thereof were tried without success in removing membranes. Although prolonged exposure to urea or pronase did remove the FM, further development was inhibited. See Berg (1967) for a review of methods of FM removal.

TABLE I

Removal of the fertilization membrane. Fertilized eggs were treated with the reagents listed under Treatment; the duration of exposure is noted under Time; the Results indicate whether the FM remained (+) or was removed (−) following treatment

Treatment	Time (post fertilization)	Results
Sucrose (0.87 M), 15°C	8 minutes–24 hr	+
Sucrose (0.87 M) + EDTA (1 mM), 15° C	7 minutes–$1\frac{3}{4}$ hr	±
Sucrose (0.87 M) + EDTA (10 mM), 15° C	10–20 minutes	− (no division by 7 hr)
Sucrose (0.87 M) + EDTA (5 mM), 2° C	10 minutes–$2\frac{1}{2}$ hr	+
Sucrose (0.87 M) + EDTA (5 mM), 15° C	10–20 minutes	− (good development)
Sucrose (p.87 M) + EDTA (5 mM), 15° C—unfertilized eggs in FSW at 2° C for 6 hr. prior to fertilization	10–40 minutes	+

OBSERVATIONS

Removal of the FM

A non-electrolyte (0.87 *M* sucrose) in combination with a chelating reagent (5 m*M* EDTA) proved to be effective both in removing the FM of freshly fertilized *Urechis* eggs (10–25 minutes postfertilization) or blastulae, and in allowing development to ensue. As can be seen from Table I, too high a concentration of EDTA (10 m*M*) led to FM$^-$ eggs which did not continue development, while too low a concentration (1 m*M* EDTA) removed the FM of only a small proportion of the eggs. The FM appeared to be solubilized at about the same rate over its entire surface. The temperature of incubation in SE proved to be important, 15° C being effective while 2° C was not. In addition, if unfertilized eggs were held at 2° C for 6 hours, though they fertilized and developed well, their FMs were resistant to dissolution.

Development of embryos lacking FM

Through the 4-cell stage, FM⁻ embryos lagged by 5–10 minutes, thereafter the embryological development of membraneless and control embryos seemed equivalent as far as rate of development and morphology of developmental stages (Figs. 1a and b). Cleavage planes were the same in control and experimental embryos for the period studied, *i.e.,* through the 16-cell stage. FM⁻ embryos were able to form respectable blastulae which began swimming shortly after 9 hours. Culture dishes of demembranated embryos always contained a small number of isolated cells. Gastrulation began at 17 to 17½ hours in control and experimental embryos. Both control and FM⁻ embryos exhibited a concentration of red pigment granules (hematin (Baumberger and Michaelis, 1931)) on the surface around the blastopore. Generally, the diameter of the blastopore of an FM⁻ early gastrula was 1.5 times that of the control. By 21 hours, the embryos were well-defined gastrulae. At this time a small population of green pigment granules was present on the surface below the prototroch over the region of the future intestine. Newby (1940) states that *Urechis* does not possess green pigments though two other species of echiuroids do (*Thalassema* and *Echiurus*). By 25 hours, both experimental and control embryos possessed a tuft of long apical cilia and the appropriate divisions of the gut. At this time, one difference in these embryos had become quite apparent, the membraneless embryos were more elongate (Fig. 1c and d). This difference was more strikingly expressed as development proceeded. At the level of the prototroch in the trochophore larva (43 hours), the width of the experimental embryo was typically only about 0.7 while its longitudinal axis was 1.3 times that of the control embryo (Fig. 1e and f). By two days, FM⁻ and control embryos were feeding and able to contract along their longitudinal axis, notably changing their shapes. Newby (1940) observed such contraction at four days of development. An unexpected difference between FM⁻ and control embryos was that at all swimming stages, the membraneless embryos were less motile than the corresponding control embryos. Unless noted, the embryology described above agrees with that presented in Newby's monograph on *Urechis* development (Newby, 1940).

Lastly, cultures of FM⁻ embryos always contained some double-embryos whereas the controls did not. Sometimes these embryos were merely "joined" at some point, usually at the pretrochal level; more often, their apical ends were "fused," there being a digestive system at each end (Fig. 1g). It may be inferred that one function of the FM is, so to speak, to keep an embryo unto itself. This same phenomenon of double embryos has been observed in the sea urchin (Vacquier and Mazia, 1968b).

From the above observations, it was concluded that removal of the FM does not interfere with normal embryogenesis in terms of such events as blastulation, gastrulation, ciliation or pigmentation, but does have some influence upon the motility, shape and "oneness" of these embryos. Biochemical data suggest that FM⁻ embryos are normal in their time of initiation of synthesis of rRNA (Engstrom, in preparation).

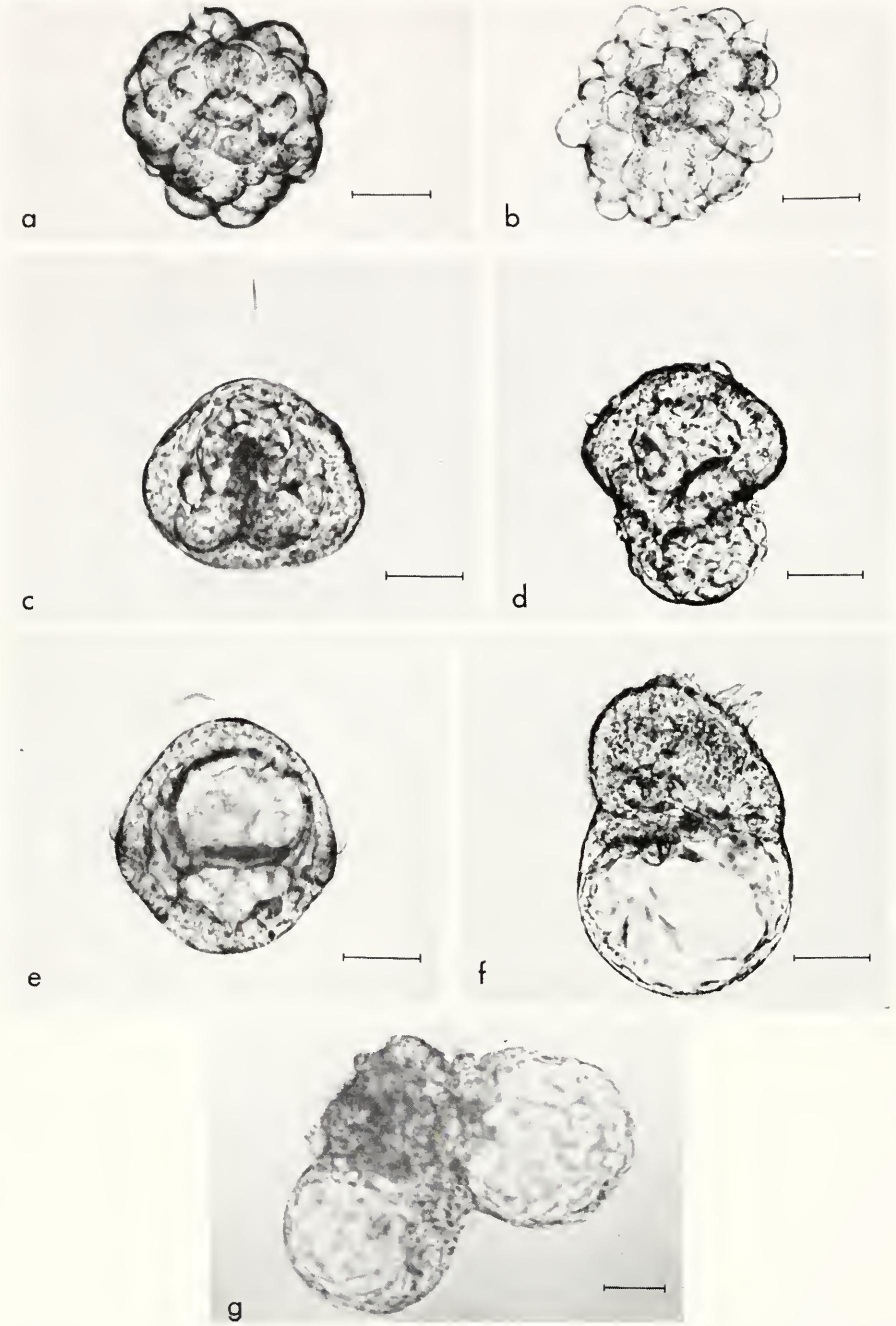

FIGURE 1. *Urechis* embryos as viewed by light microscopy: (a.) 6¾ hour FM$^+$, apical view; (b.) 7¼ hour FM$^-$, apical view; (c.) 24 hour FM$^+$, latero-posterior view; (d.) 24 hour FM$^-$, latero-anterior view; (e.) 46 hour FM$^+$, lateral view; (f.) 46 hour FM$^-$, lateral view; (g.) "Double" embryos (FM$^-$) at 4 days as viewed by light microscopy; lateral view. Bar on figure represents 50 μ.

DISCUSSION

Mechanism of membrane removal

In hindsight, it was not surprising that a nonelectrolyte along with a chelating agent would remove the FM from a marine embryo which appears to retain its FM into late development. The necessity to invoke the use of a chelating agent in addition to sucrose suggests that ions, especially divalent cations, play a role in the integrity of the FM of *Urechis* eggs. These findings are in line with Temin's (1956) proposal that the FM of the *Urechis* egg is composed of two layers, an outer protein layer, soluble in nonelectrolyte, and an inner calcium-proteinate layer. In addition, the removal of the FM from unfertilized eggs of *S. purpuratus* with non-electrolyte can be prevented by the addition of ions, particularly divalent cations (Dean and Moore, 1949; Moore, 1932).

The sensitivity of membrane removal to temperature may mean that by 2½ hours EDTA had not yet had access to the Ca-proteinate layer. It is possible, though not to be expected, that Ca^{++} ion deposition in the membrane was greater or the bonding more tenacious at lower temperature. Unfertilized eggs seem to "age" and render the FM more resistant to the action of SE. It is difficult to account for this finding but, since the eggs were not observed for a long period of time, it is possible the FM would have eventually dissolved. Resistance to treatment cannot have been due to the usual "hardening" process since the FM can be removed at least as late as the blastula stage.

The unexpected difference in the degree of motility between the membraneless and control embryos might be explained by supposing that the FM has an energy-providing role. Since the cilia pass through the FM this seems a reasonable hypothesis. A second alternative is that some component(s) of the cilia may have been solubilized or inactivated during exposure of the embryo to SE. It has been shown that some components of isolated cilia of *Tetrahymena* are solubilized in low ionic strength-EDTA buffer (Renaud, Rowe and Gibbons, 1968). Since SE treatment of *Urechis* embryos preceded the appearance of cilia by at least 7 hours, the above speculation presumes: (1) the existence of a pool of ciliary proteins and (2) that the effect of this nonelectrolyte-chelating solution (SE) would be at the level of ciliary protein units or subunits. Evidence for a pool of ciliary proteins has been reported by Auclair and Siegel (1966). Lastly, the present observations cannot exclude the possibility that FM^- embryos simply have fewer cilia and so are less motile than FM^+ embryos.

Development of membraneless embryos

Up through the trochophore stage, the membraneless embryos appeared normal in their embryological development though abnormal in shape. This difference was first detected at the beginning of gastrulation. Recall that the blastopore of the FM^- embryo was larger than that of the normal embryo and that older experimental embryos were longer and narrower than controls. The explanation for the earlier observation is probably that during invagination, the FM being relatively inelastic, acts as a restraint against outward directed cell movements. One would expect this lack of restraint to be reflected in the overall diameter of the blastula, especially in the vegetal region. At about 48 hours of development, the archenteron of the control embryo has undergone distention and stretching. By this time, cuticle formation has also begun (Newby, 1940). Without the restraining influence of the FM, this distention of the archenteron might cause the membraneless embryo to become more elongate. One can conclude that there was no cuticle formation to prevent this shape change in FM^- embryos. The importance of the FM for the shape of the embryos suggests that the FM is retained into later development. Support for this point comes from studies on worms from another phylum, Sipunculoidea. First it should be recalled that unlike sea urchins, *Urechis* blastulae do not shed the FM when they "hatch," rather the cilia pass through the FM. This same behavior is seen in three different genera of sipunculids; further, the FM persists in the sipunculid trochophore and is transformed into the cuticle of the metamorphosing larva (Rice, 1967).

Further, either a hyaline layer is normally absent, removed by nonelectrolyte, or if present, is unimportant in influencing the shape of the embryo. Circumstantial evidence suggests that there is no hyaline layer in FM^- embryos. Moore (1930) found that pretreatment of unfertilized *S. purpuratus* eggs with nonelectrolyte prevented the formation of a FM or hyaline layer upon fertilization. A more direct kind of evidence is that FM^- *Urechis* embryos were able to form double embryos. This has also been found with sea urchin embryos which are lacking a hyaline layer (Vacquier and Mazia, 1968b). Assuming that no hyaline layer is present in FM^- embryos, one can infer that intercellular bonding between blastomeres is important in the maintenance of structure of the embryos. This is similar to the case in the sand dollar, *Dendraster excentricus*, in which cell-hyaline layer interaction is weak with intercellular bonding assuming the dominant role in binding blastomeres together (Vacquier and Mazia, 1968a). In summary, it may be that the FM and intercellular bonding are important in maintaining the shape of an *Urechis* embryo.

SUMMARY

It has been shown that the fertilization membrane of the *Urechis* embryo can be removed by treatment with a sucrose-EDTA solution. The resulting membraneless embryos appear to undergo nearly normal embryogenesis, differing from the controls only in shape, degree of motility and ability to form double-embryos.

LITERATURE CITED

AUCLAIR, W., AND B. SIEGEL, 1966. Cilia regeneration in the sea urchin embryo: Evidence for a pool of ciliary proteins. *Science*, **154**: 913–915.

BAUMBERGER, J. P., AND L. MICHAELIS, 1931. The blood pigments of *U. caupo*. *Biol. Bull.*, **61**: 417–421.

BERG, W., 1967. Some experimental techniques for eggs and embryos of marine invertebrates. Pages 767–776 in F. H. Wilt and N. K. Wessels, Eds., *Methods in Developmental Biology*. Crowell, New York.

DEAN, R. B., AND A. R. MOORE, 1949. The protective action of cations on the membrane forming capacity of echinoderm eggs. *Biodynamics*, **6**: 213–216.

GIUDICE, G., V. MUTOLO AND A. MOSCONA, 1967. Synthesis of ribosomal RNA during sea urchin development. *Biochim. Biophys. Acta*, **138**: 276–285.

GOULD, M., 1967. Echiuroid worms: *Urechis*. Pages 163–171 in F. H. Wilt and N. K. Wessels, Eds., *Methods in Developmental Biology*. Crowell, New York.

MOORE, A. R., 1930. Fertilization and development without membrane formation in the egg of the sea urchin, *Strongylocentrotus purpuratus*. *Protoplasma*, **9**: 9–17.

MOORE, A. R., 1932. The dependence of cytoplasmic structures in the egg of the sea urchin on the ionic balance of the environment. *J. Cell. Comp. Physiol.,* **2**: 41–51.

NEWBY, W., 1940. The embryology of the echiuroid worm, *Urechis caupo. Memoirs, American Philosophical Society, Philadelphia,* **16**: 1–219.

PFOHL, R. J., AND G. GIUDICE, 1967. The role of cell interaction in the control of enzyme activity during embryogenesis. *Biochim. Biophys. Acta,* **142**: 263–266.

RENAUD, F. L., A. J. ROWE AND I. R. GIBBONS, 1968. Some properties of the protein forming the outer fibers of cilia. *J. Cell Biol.,* **36**: 79–90.

RICE, M., 1967. A comparative study of the development of *Phascolosoma agassizii, Golfingia pugettensis* and *Themiste pyroides* with a discussion of developmental patterns in sipuncula. *Ophelia,* **4**: 143–171.

SCONZO, G., A. M. PIRRONE, V. MUTOLO AND G. GIUDICE, 1970. Synthesis of ribosomal RNA in disaggregated cells of sea urchin embryos. *Biochim. Biophys. Acta,* **199**: 441–446.

SHIOKAWA, K., AND K. YAMANA, 1967. Pattern of RNA synthesis in isolated cells of *Xenopus laevis* embryos. *Develop. Biol.,* **16**: 368–388.

TEMIN, H., 1956. Studies of activation in eggs of *Urechis caupo, Nereis limbata* and *Asterias forbesi. Biol. Bull.,* **111**: 313.

VACQUIER, V., AND D. MAZIA, 1968a. Twinning of sand dollar embryos by means of dithiothreitol (Structural basis of blastomere interactions). *Exp. Cell Res.,* **52**: 209–221.

VACQUIER, V., AND D. MAZIA, 1968b. Twinning of sea urchin embryos by treatment with dithiothreitol. *Exp. Cell Res.,* **52**: 459–468.

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COMPARISON OF CHROMATOPHOROTROPINS FROM THE HORSESHOE CRAB *LIMULUS POLYPHEMUS*, AND THE FIDDLER CRAB, *UCA PUGILATOR*[1]

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Chromatophore responses of fiddler crabs have been utilized to study the chromatophorotropins that these crabs produce themselves and, in addition, to assay for chromatophorotropic substances from other species of invertebrates. Brown and Cunningham (1941) found that extracts of the central nervous system (CNS) of the horseshoe crab, *Limulus polyphemus,* cause dispersion of the pigment in the melanophores of the fiddler crab, *Uca pugnax,* and concentration of the pigment in its leucophores. The melanin-dispersing and white pigment-concentrating responses evoked by the seven portions of the CNS tested paralleled each other so closely that Brown and Cunningham suggested both effects may have been caused by a single substance. More recently, Rao and Fingerman (1970a) investigated the chromatophorotropic activity of extracts of radial nerves from the sea star, *Asterias amurensis,* and found that after gel filtration certain fractions which caused melanin dispersion in the fiddler crab, *Uca pugilator,* also evoked pigment concentration in the leucophores and erythrophores. Extracts of horseshoe crab CNS have not been tested on erythrophores of fiddler crabs. On the other hand, extracts of the eyestalks of the fiddler crab, *Uca pugilator,* have been clearly shown to contain substances that will cause dispersion of the pigment in its melanophores, leucophores and erythrophores and concentration of the pigment in its leucophores and erythrophores (Carlson, 1935; Sandeen, 1950; Brown, 1950; Rao, Fingerman and Bartell, 1967). Differential solubility analyses and gel filtration studies of chromatophorotropins from the eyestalks of the fiddler crab, *Uca pugilator,* indicate that each chromatophorotropin has either a pigment-dispersing or pigment-concentrating action but not both (Rao, Fingerman and Bartell, 1967; Fingerman and Couch, 1967; Fingerman and Rao, 1969a, 1969b). The present investigation was undertaken (a) to determine whether the active material in the CNS of the horseshoe crab has the same elution pattern from Bio-Gel P-6 and evokes the same chromatophoric responses in the fiddler crab as did that from the radial nerves of the sea star and (b) to compare some of the properties of chromatophorotropins from the CNS of the horseshoe crab and the eyestalks of the fiddler crab.

MATERIALS AND METHODS

The horseshoe crabs, *Limulus polyphemus,* were collected in the vicinity of Woods Hole, Massachusetts, by members of the Supply Department of the Marine Biological Laboratory. We wish to express our appreciation for their efforts. The fiddler crabs, *Uca pugilator,* were obtained from Panacea, Florida. The total

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length of the horseshoe crabs used in this investigation was 105–125 mm; carapace width of the fiddler crabs was 16–19 mm.

The extracts were assayed on the melanophores, erythrophores and leucophores of the fiddler crab. The melanin and red pigment of eyestalkless fiddler crabs was maximally concentrated; the red pigment of intact fiddler crabs in black containers is maximally dispersed; and the white pigment of intact Panacea fiddler crabs in a black container is maximally concentrated. The white pigment of eyestalkless Panacea fiddler crabs is found in all stages ranging from maximal concentration to maximal dispersion. Eyestalkless individuals whose white pigment was maximally dispersed were selected for use in these experiments. Therefore, by making use of intact and eyestalkless fiddler crabs assays could be made for melanin-dispersing, red pigment-dispersing, white pigment-dispersing, red pigment-concentrating and white pigment-concentrating substances. The Hogben and Slome (1931) method was used to stage the chromatophores on the anteroventral surface of the second or third walking leg. According to this system stage 1 represents maximal concentration of the pigment, stage 5 maximal dispersion and stages 2, 3 and 4 the intermediate conditions. These chromatophore stages were used to calculate Standard Integrated Responses (SIR) to extracts as defined by Fingerman, Rao and Bartell (1967). The SIR is a measure of both the amplitude and duration of the response.

Extracts of the tissues to be assayed, the CNS of the horseshoe crab and the eyestalks of the fiddler crab, were prepared in several different ways depending upon the aim of the experiment. The particular method used will be presented in the appropriate place below as the experiments are described. In those gel filtration experiments where distilled water was used to elute the active material from the column, each fraction was mixed with an appropriate volume of 400% physiological saline (Pantin, 1934) to provide an extract that was isosmotic with the blood of the fiddler crab. Otherwise 100% saline was used. The injected dose was 0.05 ml per crab. Control crabs received 100% saline alone. Each fraction obtained from the gel chromatography experiments was assayed on three crabs; all other extracts were assayed on five crabs.

Experiments and Results

Comparison of the chromatophorotropic activity of the ethanol-soluble fraction, the 95% methanol:chloroform (2:1)-soluble fraction and a saline extract of the central nervous system of the horseshoe crab

The first objective of this series of experiments was to assay extracts of the horseshoe crab CNS prepared directly in saline on the melanophores and leucophores of a fiddler crab as did Brown and Cunningham (1941), but *Uca pugilator* would be used instead of the species, *Uca pugnax*, they used. In addition the effect of such an extract on the erythrophores of a fiddler crab would also be determined for the first time. Secondly, it has been demonstrated that the ethanol-soluble fraction (Rao, Bartell and Fingerman, 1967) and the methanol:chloroform-soluble fraction (Bartell, Rao and Fingerman, 1971) of the eyestalks of the fiddler crab, *Uca pugilator*, prepared in concentrations higher than 0.3 eyestalk equivalent per dose evoke much stronger melanin-dispersing responses than do saline extracts but below this concentration the differences among the responses to such extracts are slight. However, a high activity ethanol-soluble fraction is not obtained when freeze-dried eyestalks are used (Rao, Bartell and Fingerman, 1968). For comparative purposes melanin-dispersing and white pigment-concentrating responses evoked by the ethanol-soluble and methanol:chloroform-soluble fractions of the horseshoe crab CNS would be compared with the responses to the saline extract to learn whether the chromatophorotropic material in the horseshoe crab CNS behaves in a similar manner to the melanin-dispersing material in the eyestalk of the fiddler crab. The extracts used in this particular set of experiments were prepared in a final concentration of 0.1 equivalent of the CNS of the horseshoe crab per dose

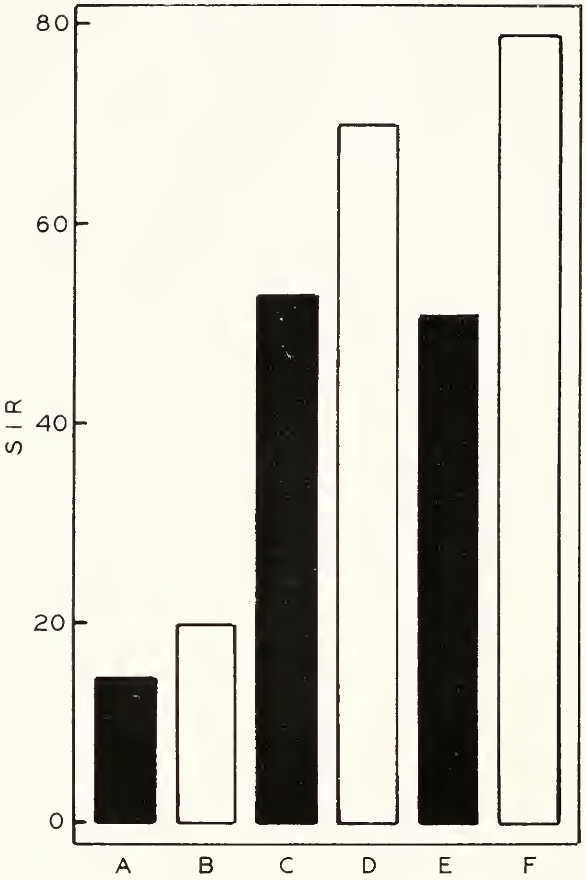

FIGURE 1. The Standard Integrated Responses (SIR) of eyestalkless fiddler crabs to extracts of the central nervous system of the horseshoe crab; solid bars, melanin-dispersing responses; empty bars, white pigment-concentrating responses. A, B, responses to extract prepared by triturating the tissue directly in saline; C, D, responses to the ethanol-soluble fraction; E, F, responses to the 95% methanol:chloroform (2:1)-soluble fraction.

of 0.05 ml. The first extract was made by triturating three freshly dissected CNS in 1.5 ml saline which provided the desired final concentration. Before injection the extract was centrifuged for three minutes at 1815 × *g* and at 24° C. The ethanol-soluble fraction was obtained by extracting three freshly dissected CNS in

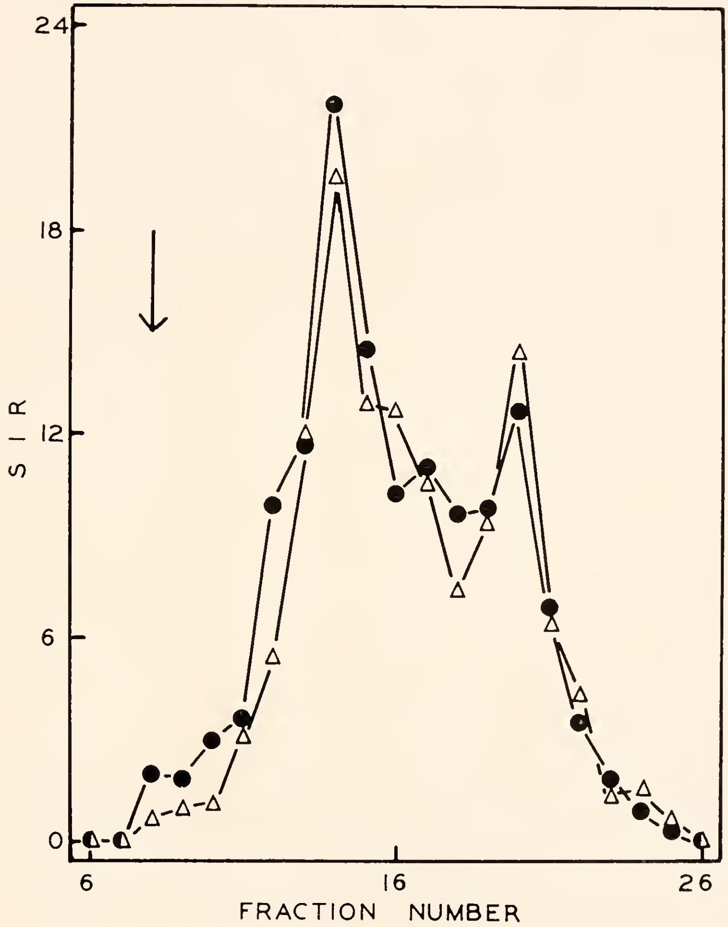

FIGURE 2. The melanin-dispersing (dots) and white pigment-concentrating (triangles) Standard Integrated Responses (SIR) of eyestalkless fiddler crabs evoked by the fractions obtained by passing an extract of the central nervous system of the horseshoe crab through a column of Bio-Gel P-6, arrow, void volume.

a total of 10 ml of 100% ethanol. This extract was then centrifuged in the same manner as was the saline extract and the clear supernatant was poured into a porcelain dish and allowed to evaporate on a heating table set at 35–40° C. When the fraction was dry the material was then suspended in 1.5 ml of saline to obtain the desired final concentration. The third extract was prepared in the same manner as was the ethanol-soluble fraction, the sole difference being that 95% methanol: chloroform (2:1) was substituted for the 100% ethanol.

The averaged results of this group of experiments, performed twice, are presented as SIR's in Figure 1. It is evident that the CNS of the horseshoe crab possesses melanin-dispersing and white pigment-concentrating activities as first reported by Brown and Cunningham (1941). However, the extracts had neither a pigment-dispersing nor pigment-concentrating effect on the erythrophores of the fiddler crab. It is clear from examination of Figure 1 that not only did the ethanol-soluble and 95% methanol:chloroform (2:1)-soluble fractions of the horseshoe crab CNS evoke both melanin-dispersing and white pigment-concentrating responses but with each of the two fractions the melanin-dispersing and white pigment-concentrating responses were more than three times as large as the corresponding responses obtained with the extract prepared directly in saline. The larger SIR's seen with the ethanol-soluble and 95% methanol:chloroform (2:1)-soluble fractions were due to the fact that these two fractions evoked responses that had both larger amplitudes and longer durations than did the responses to the extract prepared directly in saline. The melanin-dispersing responses generated by the saline extract, the ethanol-soluble fraction and the 95% methanol:choroform (2:1)-soluble fractions showed peak chromatophore stages of 3.1, 4.5 and 4.7 respectively, and the responses lasted 5.5, 12.5 and 15.0 hours respectively. The corresponding values for the white pigment-concentrating responses where the initial stage of the pigment was 5 were chromatophore stages of 2.2, 1.3 and 1.1, and the responses lasted 6, 13 and 16 hours respectively. An hypothesis of Bartell, Rao and Fingerman (1971) which is based in part on the differences in the physical state of the material in saline, ethanol and methanol:chloroform extracts of eyestalks from the fiddler crab and which explains these differences in the SIR's will be discussed below.

Gel filtration of extracts of the central nervous system of the horseshoe crab and eyestalks of the fiddler crab

The objectives of this experiment were to attempt to separate the melanin-dispersing activity from the white pigment-concentrating activity in the horseshoe crab CNS by means of gel filtration and to compare the rates of flow (R_f) for these activities with that of the material extracted from the radial nerves of the sea star and the eyestalks of the fiddler crab. R_f is defined as the quotient of the fraction void volume/elution volume. In the first experiment of this series 10 CNS were triturated in 0.5 ml distilled water and centrifuged as described above for the saline extract. The supernatant was then applied to the top of a column of Bio-Gel P-6 whose dimensions were 57 × 0.9 cm and eluted with distilled water. The void volume was 16 ml and the flow rate was 38 ml/hr. Two ml fractions were collected. The averaged results from a total of four chromatographic separations are presented in Figure 2. It is evident from examination of this figure that the elution pattern of the melanin-dispersing activity was virtually the same as that of the white pigment-concentrating activity. The major peak for both activities occurred in fraction 14 and had an R_f of 0.57. The averaged data for both activities showed a secondary peak in fraction 20 with an R_f of 0.4. It should be noted, however, that whereas the R_f 0.57 peak was a consistent feature in every experiment, the secondary peak was observed in only three of the four experiments. The secondary peak at R_f 0.4 may represent an additional chromatophorotropic substance or substances.

Another gel, Sephadex LH-20, was then tried in an effort to separate the melanin-dispersing and white pigment-concentrating activities. The gel was prepared in 95% methanol:chloroform (2:1) which was also used as the elution medium and the solvent for extraction of the active material. Five freshly dissected CNS were extracted in 0.3 ml of the solvent, centrifuged as above and the supernatant was applied to the Sephadex LH-20 column whose dimensions were 28 × 0.7 cm. There is, of course, some dilution of the solvent because of the water in the tissue. The flow rate was 20 ml/hr and 1 ml fractions were collected. After the solvent had evaporated the residue from each fraction was suspended in

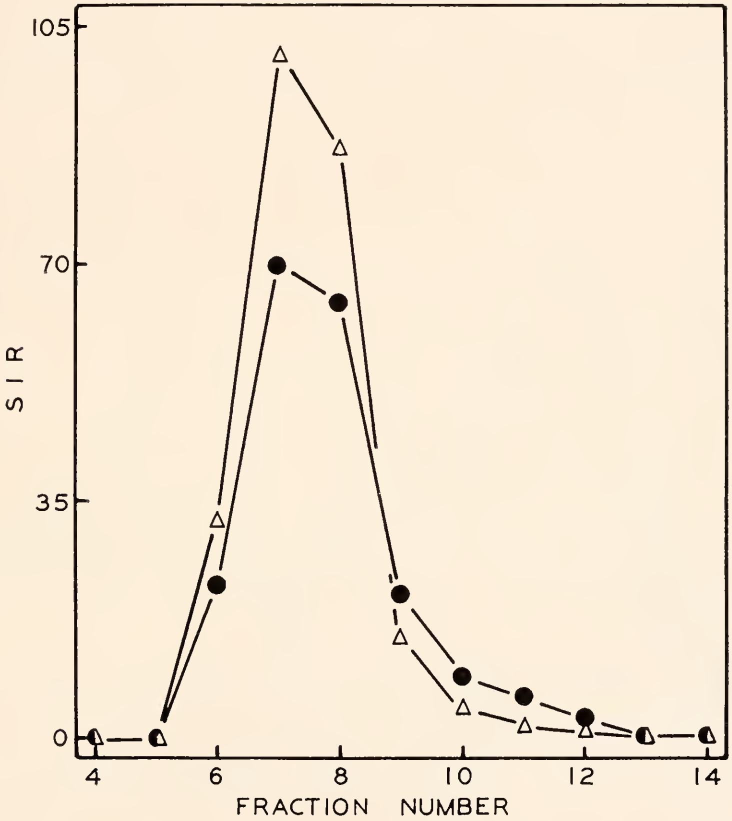

FIGURE 3. The melanin-dispersing (dots) and white pigment-concentrating (triangles) Standard Integrated Responses (SIR) of eyestalkless fiddler crabs evoked by passing the 95% methanol:chloroform (2:1)-soluble fraction extract of the central nervous system of the horseshoe crab through a column of Sephadex LH-20.

0.3 ml 100% saline and assayed. This experiment was performed twice and the averaged results are presented in Figure 3. It is clear from inspection of this figure that once again the melanin-dispersing and white pigment-concentrating activities did not separate from each other.

Although some chromatophorotropins from the fiddler crab have been analyzed previously by gel filtration (Fingerman and Couch, 1967; Fingerman and Rao, 1969a, 1969b), there has been no previous attempt to assay the several fractions simultaneously on the melanophores, leucophores and erythrophores. Consequently, definite conclusions concerning the elution patterns of the chromatophorotropins active on these three types of chromatophore were not possible. It was necessary to assay the fractions from the same chromatographic separation for several chromatophorotropic activities simultaneously if we were to attempt to determine the number of chromatophorotropins in the eyestalks of this fiddler crab. The resulting data could then be compared with those obtained (Fig. 2) after gel filtration of the extracts of the horseshoe crab CNS. The void volume of the column used to fractionate eyestalk extracts was 40 ml; the volume of each

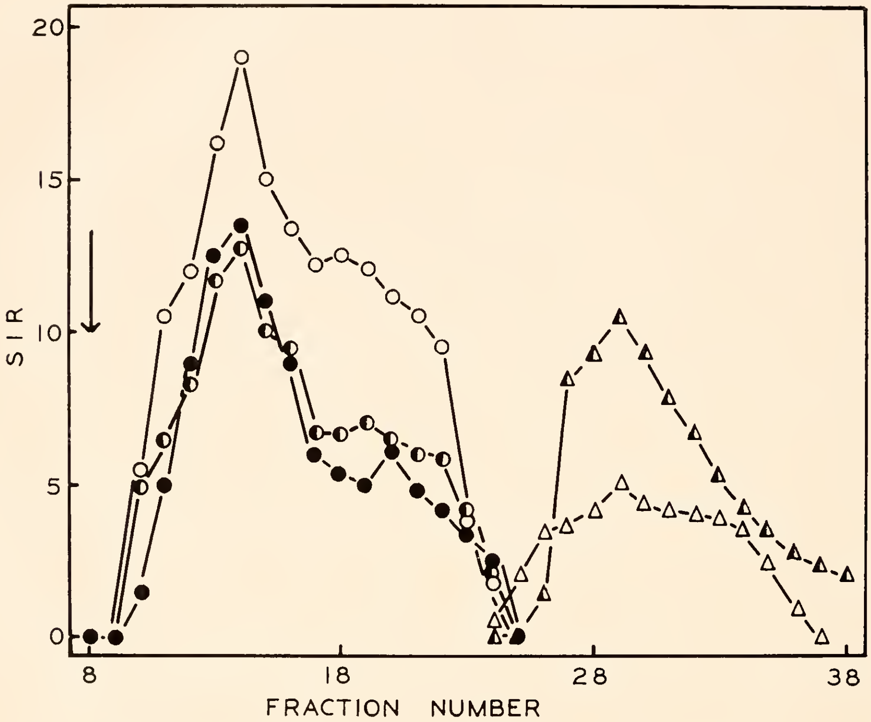

FIGURE 4. The Standard Integrated Responses (SIR) of fiddler crabs evoked by the fractions obtained by passing an extract of fiddler crab eyestalks through a column of Bio-Gel P-6; dots, melanin-dispersing responses; circles, white pigment-dispersing responses; half-filled circles, red pigment-dispersing responses; empty triangles, white pigment-concentrating responses; half-filled triangles, red pigment-concentrating responses, arrow, void volume.

fraction collected was 5 ml. A lyophilized distilled water extract of 300 eyestalks was dissolved in 1.5 ml distilled water and applied to a 71 × 1.5 cm column of Bio-Gel P-6 equilibrated and eluted with distilled water at a flow rate of 25 ml/hr. The several fractions generated not only melanin dispersion and white pigment concentration, but white pigment dispersion, red pigment dispersion and white pigment concentration as well. The averaged SIR's for the several chromatophorotropic responses are plotted in Figure 4 *versus* the fraction number. This experiment was performed twice. The pigment-concentrating material was separated by this gel from the pigment-dispersing material. The former had a peak at R_f 0.28 and the latter at 0.57. However, the three pigment-dispersing activities did not separate from each other. Likewise, the two pigment-concentrating activities did not separate from each other.

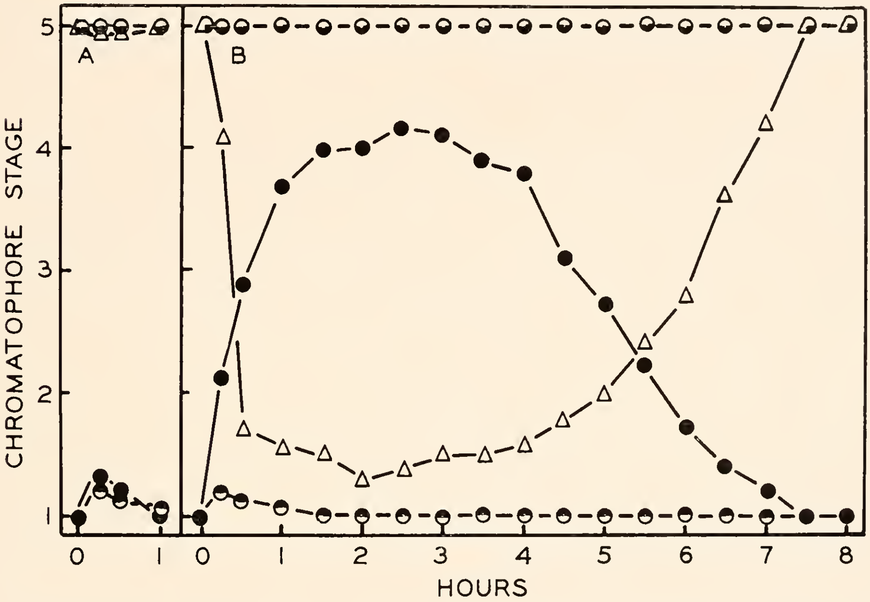

FIGURE 5. Relationships between chromatophore stage of eyestalkless fiddler crabs and time following injection of the (A) acetone-soluble and (B) acetone-insoluble fractions of the central nervous system of the horseshoe crab; dots, melanophores of crabs that received one of the fractions; triangles, white chromatophores of crabs that received one of the fractions; circles half-filled on top, melanophores of control crabs; circles half-filled on bottom, white chromatophores of control crabs.

Test for acetone solubility of the chromatophorotropic material in the central nervous system of the horseshoe crab

The melanin-dispersing material from the fiddler crab, *Uca pugilator*, is insoluble in acetone (Abramowitz and Abramowitz, 1938) whereas the white pigment-concentrating substance of this crab is soluble in acetone (Rao, Fingerman and Bartell, 1967). Furthermore, the latter investigators were able to separate the white pigment-dispersing and pigment-concentrating substances of this fiddler crab from each other by acetone extraction, the white pigment-dispersing substance being insoluble in acetone just as is the melanin-dispersing material. The present experiment was designed to determine whether the melanin-dispersing and white pigment-concentrating activities of the horseshoe crab CNS could be separated from each other by acetone extraction and if not then to determine whether the chromatophorotropic material from the horseshoe crab resembles the pigment-dispersers or the white pigment-concentrator of the fiddler crab with respect to acetone solubility.

The experiment, performed twice, was carried out in the following manner in a dehumidified room. Two CNS of the horseshoe crab were freeze-dried and then extracted three times with a total of 10 ml of acetone. The extract was centrifuged as above, the supernatant was poured into an evaporating dish and the acetone was allowed to evaporate on the warming stage. In similar fashion the insoluble material was dried. The dried acetone-soluble and acetone-insoluble fractions were then suspended in 1 ml of saline to provide a final concentration of 0.1 CNS equivalent per dose and assayed for melanin-dispersing and white pigment-concentrating activities. The average chromatophore stages are shown in Figure 5 where it can be seen that activity was present only in the acetone-insoluble fraction.

Discussion

These experiments have confirmed the observation of Brown and Cunningham (1941) that the CNS of the horseshoe crab evokes melanin-dispersion and white concentration in fiddler crabs. But whereas the fiddler crab, *Uca pugilator,* produces both red pigment-dispersing and pigment-concentrating substances (Brown, 1950), extracts of the horseshoe crab CNS have no effect on the erythrophores of this fiddler crab. The active material in the horseshoe crab CNS differs from that found in the radial nerve of the sea star by Rao and Fingerman (1970a) in the following ways. The latter material not only dispersed the melanin and concentrated the white pigment as did the horseshoe crab material, but in addition it concentrated the red chromatophoric pigment. Also, whereas the major peaks of activity for both melanin dispersion and white pigment concentration obtained after gel filtration of aqueous extracts of the horseshoe crab CNS both had an R_f of 0.57 on Bio-Gel P-6 (Fig. 2), the three activities from the sea star had an R_f of 0.39 which indicates that the chromatophorotropic material from the sea star has a smaller size. Although the material comprising the secondary peak, R_f 0.4, obtained with the horseshoe crab CNS extract on Bio-Gel P-6 is very close to that of the material in the sea star radial nerves (R_f 0.39), these materials nevertheless differ in their capacity to evoke a response of the erythrophores. The lack of response of the erythrophores could not have been due simply to a difference in the amount of horseshoe crab material injected as compared with that from the sea star. The SIR's of the melanophores and leucophores to the horseshoe crab material (Fig. 1A, B) were larger than the corresponding SIR's obtained with the fractions of the radial nerves (Rao and Fingerman, 1970a), and yet there was no response of the erythrophores to the horseshoe crab extract.

The R_f values for the pigment-dispersing and pigment-concentrating substances of the fiddler crab were 0.57 and 0.28, respectively (Fig. 4), indicating thereby that the pigment-concentrating activities are due to material of smaller size than are the pigment-dispersing activities. The sea star material with an R_f of 0.39 is apparently intermediate in size between them. On the other hand, the major peaks for both the melanin-dispersing and white pigment-concentrating activities of the horseshoe crab had the same R_f (0.57) as that of the pigment-dispersing activities of the fiddler crab. Whereas in the fiddler crab the pigment-dispersing and pigment-concentrating activities are clearly due to different molecules, both activities in the extracts from the CNS of the horseshoe crab might be due to the same molecule. If not the same molecule, then both activities are caused by different substances having similar elution patterns from Bio-Gel P-6. Just as with Bio-Gel P-6, chromatography on the gel Sephadex LH-20 failed to separate the two chromatophorotropic activities in the horseshoe crab CNS (Fig. 3). The possibility that pigment-dispersing and pigment-concentrating responses are both due to a single substance in the horseshoe crab CNS is not without precedent. The intermedins not only cause melanin dispersion in frog skin but concentration of the pigment in the guanophores as well (Bagnara, 1964).

The chromatophorotropic material in the horseshoe crab CNS is more closely related to the melanin-dispersing material of the fiddler crab than to the white pigment-concentrating hormone of the fiddler crab in three ways: (a) both the melanin-dispersing material of the fiddler crab and the material from the horseshoe crab that evokes melanin dispersion and white pigment concentration are insoluble in acetone (Abramowitz and Abramowitz, 1938; Fig. 5) but the white pigment-concentrating hormone of the fiddler crab is acetone-soluble (Rao, Fingerman and Bartell, 1967), (b) the melanin-dispersing material of the fiddler crab and the chromatophorotropic material from the horseshoe crab were eluted with an R_f of 0.57 (Figs. 2 and 4) from a column of Bio-Gel P-6 whereas the white pigment-concentrating hormone had an R_f of 0.28; and (c) whereas ethanol and methanol: chloroform can be used to obtain extracts of the horseshoe crab CNS (Fig. 1) and melanin-dispersing material from the eyestalks of the fiddler crab (Rao, Bartell and Fingerman, 1967; Bartell, Rao and Fingerman, 1971) which are more potent than extracts prepared directly in saline, the white pigment-concentrating hormone in these eyestalks does not behave in the same manner (Fingerman and Rao, 1969a).

As noted above, although the pigment-dispersing activities from the eyestalks of the fiddler crab could be separated from the pigment-concentrating activities, the three pigment-dispersing activities could not be separated from each other nor could the two pigment-concentrating activities be separated from each other on Bio-Gel P-6 (Fig. 4). Similarly, the pigment-dispersing activities in the eyestalks of the prawn, *Pandalus jordani*, could not be separated from each other by the chromatographic techniques that Kleinholz (1970) used. The respective pigment-dispersing and pigment-concentrating activities seen in Figure 4 are each caused by (a) a single molecule or (b) different substances having similar elution patterns from Bio-Gel P-6. Indirect evidence obtained with the fiddler crab, *Uca pugilator*, tends to favor the latter alternative. For example, in eyestalkless fiddler crabs of this species from Panacea, Florida, the melanin was maximally concentrated whereas the white pigment showed all stages from maximal concentration to maximal dispersion (Rao, Fingerman and Bartell, 1967). If the chromatophores of a the fiddler crab were controlled by a single pigment-dispersing hormone and a single concentrator, then we would reasonably expect the pigments to be in lockstep, not having one fully dispersed while the other is fully concentrated. Furthermore, Rao, Fingerman and Bartell (1967) found that extracts of the circumesophageal connectives caused melanin dispersion in this crab, but did not disperse the white pigment. Fingerman and Couch (1967) using the gel Sephadex G-25 fractionated extracts of eyestalks from this fiddler crab and obtained some fractions which while evoking equal melanin-dispersing responses differed widely in their red pigment-dispersing activities. The differences were not due to antagonism by a red pigment-concentrating hormone. More recently, Rao and Fingerman (1970b) found that serotonin does not evoke melanin dispersion in this fiddler crab. But serotonin had an indirect effect on the erythrophores causing dispersion of the red pigment by stimulating release of a red pigment-dispersing substance. The occurrence of red pigment dispersion in the absence of melanin dispersion suggests that (a) different substances are involved or (b) melanin-dispersing substances also have red pigment-dispersing activity, but in addition there are discrete red pigment-dispersing substances.

Bartell, Rao and Fingerman (1971) have postulated that the high activities generated by ethanol-soluble and methanol:chloroform-soluble fractions of eyestalks from the fiddler crab are due to extraction of a micellar lipoprotein with a melanophorotropic polypeptide component. It was suggested that the lipoprotein micelles slowly release the polypetide, thereby accounting for the prolonged activity generated by these fractions. The same hypothesis can be applied to explain the prolonged responses obtained with the ethanol-soluble and 95% methanol:chloroform (2:1)-soluble fractions of the CNS of the horseshoe crab. Vigorous stirring and lyophilization of the ethanol-soluble fraction of the eyestalks dissociate the active polypeptide from the lipoprotein.

Summary and Conclusions

1. Extracts of the central nervous system of the horseshoe crab, *Limulus polyphemus*, were assayed for chromatophorotropic activity on the fiddler crab, *Uca pugilator*. The extracts caused pigment dispersion in the melanophores and pigment concentration in the leucophores but had no effect on the erythrophores.

2. The ethanol-soluble and 95% methanol:chloroform (2:1)-soluble fractions of the central nervous system from the horseshoe crab evoked melanin-dispersing and white pigment-concentrating responses which had larger amplitudes and longer durations than did the responses caused by extracts prepared directly in saline.

3. Neither gel filtration nor acetone fractionation was effective in separating the melanin-dispersing activity from the white pigment-concentrating activity in extracts of the central nervous system of the horseshoe crab. These responses appear to be caused by either the same molecule or by different substances having similar elution patterns from Bio-Gel P-6.

4. Extracts of the eyestalk of the fiddler crab were fractionated on Bio-Gel P-6. The melanin-dispersing, white pigment-dispersing and red pigment-dispersing ac-

tivities were eluted with an R_f of 0.57, the same value as that of the major peaks of melanin-dispersing and white pigment-concentrating activities from the central nervous system of the horseshoe crab. In contrast, the white pigment-concentrating and red pigment-concentrating activities of the fiddler crab separated from the pigment-dispersing activities, having been eluted later from the column of Bio-Gel P-6 with an R_f of 0.28.

5. The chromatophorotropic material in the central nervous system of the horseshoe crab is more closely related to the melanin-dispersing material of the fiddler crab than to the white pigment-concentrating hormone of the fiddler crab.

6. The chromatophorotropins in the central nervous system of the horseshoe crab and the radial nerves of the sea star differ from each other in their elution patterns from a column of Bio-Gel P-6 and in the chromatophore responses they evoke in the fiddler crab.

LITERATURE CITED

Abramowitz, A. A., and R. K. Abramowitz, 1938. On the specificity and related properties of the crustacean chromatophorotropic hormone. *Biol. Bull.*, **74**: 278–296.

Bagnara, J. T., 1964. Stimulation of melanophores and guanophores by melanophore-stimulating hormone peptides. *Gen. Comp. Endocrinol.*, **4**: 290–294.

Bartell, C. K., K. R. Rao and M. Fingerman, 1971. Comparison of the melanin-dispersing fractions in extracts prepared initially in ethanol, saline, or distilled water from eyestalks of the fiddler crab, *Uca pugilator*. *Comp. Biochem. Physiol.*, **38A**: 17–36.

Brown, F. A., Jr., 1950. Studies on the physiology of *Uca* red chromatophores. *Biol. Bull.*, **98**: 218–226.

Brown, F. A., Jr., and O. Cunningham, 1941. Upon the presence and distribution of a chromatophorotropic principle in the central nervous system of *Limulus*. *Biol. Bull.*, **81**: 80–95.

Carlson, S. P., 1935. The color changes in *Uca pugilator*. *Proc. Nat. Acad. Sci.*, **21**: 549–551.

Fingerman, M., and E. F. Couch, 1967. Differentiation of chromatophorotropins from the prawn, *Palaemonetes vulgaris*, and the fiddler crab, *Uca pugilator*. *J. Exp. Zool.*, **165**: 183–194.

Fingerman, M., and K. R. Rao, 1969a. A comparative study of leucophore-activating substances from the eyestalks of two crustaceans, *Palaemonetes vulgaris*, and *Uca pugilator*. *Biol. Bull.*, **136**: 200–215.

Fingerman, M., and K. R. Rao, 1969b. Physiology of the brown-black chromatophores of the stomatopod crustacean *Squilla empusa*. *Physiol. Zool.*, **42**: 138–147.

Fingerman, M., K. R. Rao and C. K. Bartell, 1967. A proposed uniform method of reporting response values for crustacean chromatophorotropins: the Standard Integrated Response. *Experientia*, **23**: 962.

Hogben, L., and D. Slome, 1931. The pigmentary effector system. VI. The dual character of endocrine co-ordination in amphibian colour change. *Proc. Roy. Soc. London, Series B*, **108**: 10–53.

Kleinholz, L. H., 1970. A progress report on the separation and purification of crustacean neurosecretory pigmentary-effector hormones. *Gen. Comp. Endocrinol.*, **14**: 578–588.

Pantin, C. F. A., The excitation of crustacean muscle. *J. Exp. Biol.*, **11**: 11–27.

Rao, K. R., and M. Fingerman, 1970a. Chromatophorotropic activity of extracts of radial nerves from the starfish, *Asterias amurensis*. *Z. Vergl. Physiol.*, **67**: 133–139.

Rao, K. R., and M. Fingerman, 1970b. Action of biogenic amines on crustacean chromatophores: I. Differential effect of certain indolealkylamines on the melanophores of the crabs *Uca pugilator* and *Carcinus maenas*. *Experientia*, **26**: 383–384.

RAO, K. R., AND M. FINGERMAN, 1970c. Action of biogenic amines on crustacean chromatophores, II. Analysis of the response of erythrophores in the fiddler crab, *Uca pugilator,* to indolealkylamines and an eyestalk hormone. *Comp. Gen. Pharmacol.,* **1**: 117–126.

RAO, K. R., C. K. BARTELL AND M. FINGERMAN, 1967. Relationship between the response of melanophores in the fiddler crab, *Uca pugilator,* and the concentration of eyestalk extract. *Z. Vergl. Physiol.,* **56**: 232–236.

RAO, K. R., C. K. BARTELL AND M. FINGERMAN, 1968. Solubility and stability properties of the melanin-dispersing substances in the eyestalks of the fiddler crab, *Uca pugilator. Z. Vergl. Physiol.,* **60**: 1–13.

RAO, K. R., M. FINGERMAN AND C. K. BARTELL, 1967. Physiology of the white chromatophores in the fiddler crab, *Uca pugilator. Biol. Bull.,* **133**: 606–617.

SANDEEN, M. I., 1950. Chromatophorotropins in the central nervous system of *Uca pugilator,* with special reference to their origins and actions. *Physiol. Zool.,* **23**: 337–352.

Reference: *Biol. Bull.*, **140**: 389–399. (June, 1971)

THE NEMATOCYST TOXIN OF *METRIDIUM*[1]

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At the beginning of the century, Richet (Richet and Portier, 1936) separated four pharmacologically active substances from the tissues of the sea anemone *Actinia* (= *Metridium* [Hand, 1954]). Since that time a great deal of effort has been directed toward the investigation of toxic materials occurring in coelenterates. It is now generally accepted that Richet's extracts were mixtures of several substances (Welsh, 1961), and a variety of compounds have been suggested as possible components of coelenterate toxins. Among the substances reported in toxic extracts of coelenterate tissues are histamine (Mathias, Ross and Schacter, 1960), tetramethylated ammonium bases (Welsh and Prock, 1958), serotonin (Welsh, 1960), polysaccharide substances (Phillips, 1956; Martin, 1966), and proteins of various molecular weights (Phillips and Abbott, 1958; Kline and Waravdekar, 1960; Lane, 1961; Blanquet, 1968). Several methods for obtaining isolated nematocysts have been described (Phillips, 1956; Lane and Dodge, 1958; Yanagita, 1959). Some investigators, however, have continued to use a variety of tissues, and in some cases entire animals, as a source of material presumed to originate in the nematocysts. The situation has been further complicated by the wide variety of animals used as sources of test material and for bioassay.

The pharmacological effects of coelenterate toxins have been investigated on a variety of physiological preparations including frog muscle, rat uterus and guinea pig ileum (Mathias *et al.*, 1960); mice (Lane, 1961; Martin, 1966); canine cardiovascular system (Hastings, Larsen and Lane, 1967); molluscan heart (Welsh, 1956); and crustacea (Lane, 1961; Blanquet, 1968). These studies have provided valuable clues as to the possible nature of coelenterate toxins. Unfortunately, it has been difficult to correlate pharmacological effects with specific substances known to be present in coelenterates, and the reaction of different assay preparations to the same compound have varied widely (*cf.* Welsh, 1956; Mathias *et al.*, 1960; Hastings *et al.*, 1967).

The purpose of the following study was to investigate the toxicity of material released upon discharge of isolated nematocysts from *Metridium* and to classify the principle active components of the toxin. Serotin, quaternary amines and indoles which are repeatedly mentioned in connection with cnidarian toxicity, were given particular attention.

MATERIALS AND METHODS

Sea anemones, *Metridium senile senile* Linnaeus (1767), were obtained from the vicinity of St. Andrews, New Brunswick. Isolated nematocysts were obtained by the method of Blanquet (1968). Five or six anemones (approximately 15 g live wet weight per anemone) were placed in 200 ml of 1 *M* sodium citrate, and prodded with a glass rod to induce the expulsion of acontia. After five to ten minutes, the animals were removed from the citrate, and the acontia were allowed to incubate for 12 hours at 4° C. This treatment results in the extrusion of undischarged nematocysts from the acontia threads. The citrate suspension was then passed through a nylon mesh screen (Nytex 64, B. and S. H. Thompson Ltd., Montreal) with the aid of suction to remove the acontia threads, centrifuged in the cold (4° C) at 480 × *g* for one hour to collect the nematocysts, and the supernatant fluid was decanted. The isolated nematocysts were examined microscopically in order to determine the percentage undischarged. The nematocysts were discharged by re-suspending in distilled water followed by incubation for 12 hours at 4° C. At the end of this time, a second examination was made for undischarged nematocysts. The nematocyst-fluid mixture was centrifuged in the cold (0° C) for 40 minutes at 12,100 × *g*, and the resulting supernatant lyophilized in a Virtis micro freeze-drying apparatus. The apparatus was kept refrigerated at 4° C or below during the lyophilization process. The resulting lyophilate was termed "crude toxin extract." In some cases, the pellet from the final centrifugation was homogenized in a chilled Potter-Elvejhem apparatus, centrifuged, and the supernatant lyophilized. The lyophilate so obtained was termed "crude pellet extract."

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Bioassay of extracted material was performed on crayfish, *Orconectes virilis* or *O. propinquus* obtained from streams in the vicinity of Toronto, Ontario, and from a local supply house. These species were used interchangeably, as they responded identically to crude toxin extract in early experiments.

Lyophilates of material to be assayed were dissolved in distilled water to give the desired concentration, and were injected into the hemocoel of the test animals via the base of the third walking leg. Bovine serum albumin (BSA), sea water, and sodium citrate were initially employed as controls. In later experiments, BSA only was used.

LD_{50} values were determined as follows: Groups of test animals (*O. virilis* or *O. propinquus*) were injected with doses of crude toxin extract based on the wet weight of the crayfish. Six animals were used at each dose level, and a sufficient variety of dosages used to provide at least five points in plotting the dose-response line. Deaths occurring within 24 hours were considered to have resulted from injected material. Criteria of death were lack of movement when probed and loss of tonus in the chelipeds. No surviving animals were re-used. Response data were analyzed by plotting the logarithm of dose against the percentage mortality, and fitting a straight line by regression (Snedecor and Cochran, 1967). A dose calculated to produce 50% mortality was then determined with confidence limits at the 95% probability level.

Potency of various extract fractions, relative to that of the whole crude extract, was calculated by the single dose method of Bliss (1952). In this method the mean response of two groups of test animals to a single dose level of the fraction being assayed is compared to the expected response based on the line used to calculate the LD_{50}.

Crude pellet extracts were examined for toxicity by injecting doses ranging from 100 to 750 mg/kg live body weight into six-animal test groups of crayfish *O. virilis*.

To determine the effects of heating, prolonged hydration, and repeated lyophilization, samples of crude toxin extract were rehydrated, heated at 55° C for 30 minutes and assayed on two groups of crayfish as described above. Additional samples were rehydrated and incubated at 4° C for 48 hours, after which they were assayed as for the heat-treated material. Samples of crude toxin extract were rehydrated and immediately lyophilized. The lyophilate was rehydrated and assayed as described above.

Studies of comparative toxicity were conducted on two ten-animal test groups of the fiddler crabs *Uca pugilator* and *Sesarma reticulatum* from the vicinity of Panacea, Florida, and on two six-animal test groups of the crayfish *Procambarus econfinae,* obtained from a commercial supplier in the southeastern United States. The results of these studies were compared with data from toxicity studies using *O. virilis* and *O. propinquus.*

Since protein was suspected of being responsible for part or all of the toxic activity of the crude toxin extracts, protein content of each batch was determined by the Folin method described by Lowry, Roseborough, Farr and Randall (1951), using BSA (Sigma Chemicals) as the protein standard. As a check on the accuracy of the Folin method, nitrogen content of duplicate samples of crude toxin extracts was determined by the micro-Kjeldahl method described by Meites and Faulkner (1962).

In an attempt to concentrate the toxic material, dialyzed extracts were prepared by rehydrating 200 mg samples of crude toxin extract in 20 ml distilled water and dialyzing against a single 31 volume distilled water. The dialysis system was held for 48 hours at 4° C, after which the material in the dialysis tubing was lyophilized. Cellulose dialysis tubing (Sargent S-252275A) was used. Approximate pore size was determined by dialysing solutions of glucose, sucrose, and raffinose, and analyzing for carbohydrate passage through the tubing. Protein determinations were made on each of the dialyzed extracts using the Folin method described above. The dialyzed extracts were tested for toxic activity by injecting two groups of six crayfish with doses of extract based on the body weight of the individual crayfish.

Additional 200 mg samples were rehydrated in 5 ml distilled water and dialyzed for 48 hours against a single 20 ml volume distilled water. The dialyzable and undialyzable fractions were then assayed for total nitrogen content using the micro-Kjeldahl method described above. In order to correlate dialyzable nitrogen with non-protein nitrogen, residual nitrogen was determined in solutions of 100 mg crude toxin extract which had been deproteinized by the addition of zinc sulfate and barium hydroxide according to the method of Meites and Faulkner (1962).

Further 100 mg samples were rehydrated in 2 ml distilled water and dialyzed against 20 ml distilled water for 48 hours. Dialyzable and undialyzable materials were lyophilized. These preparations were assayed on the crayfish *Procambarus econfinae.*

As a potential aid in identifying the components of the toxic material, ultraviolet absorption spectra were determined on crude toxin extract, dialyzable material and undialyzable material using a Zeiss PMQ II spectrophotometer.

Further information on the composition of the crude toxin extract and dialyzed preparations was obtained using paper chromatography on Whatman #1 filter paper. 500 μg samples of unhydrolyzed crude extract and dialyzed material were used. One dimensional chromatograms were performed using butanol-acetic acid-water (10:3:1), which proved to be more satisfactory than other solvent systems. Acetone-water (8:2) was employed as the second solvent system for two dimensional chromatograms. Dried chromatograms were scanned with ultraviolet light (Mineralight SL 2537, short wave filter) to detect absorbing or fluorescing spots. Following this, one of a variety of detection reagents was used: 0.2% ninhydrin or copper sulfate for amines, Ehrlich's reagent for indoles, Nessler's reagent for carboxylic acids, acetylacetone and p-dimethylaminobenzaldehyde for hexosamines, 1-nitroso-2-naphthal for 5-hydroxyindoles, potassium dichromate and 40% formalin for aromatic amines (Dawson, Elliot, Elliot and Jones, 1959), and Dragendorf's reagent for the detection of quaternary ammonium bases (Bregoff, Roberts and Delwiche, 1953). In addition, serotonin content was determined by the method of Udenfriend, Clark and Neissbach (1955), using serotonin creatinine sulfate (British Drug Houses) as the standard.

Since the results of paper chromatography and the presence of dialyzable nitrogen compounds made the presence of amines a strong possibility, derivatives of crude toxin extracts were prepared for possible melting point characterization. Amines were prepared as actyl derivatives by reacting crude extract with acetic anhydride, as benzenesulfonyl derivatives by reaction with benzenesulfonyl chloride, and as picrates by reaction with picric acid. Amide derivatives of carboxylic acids were obtained by reaction with thionyl chloride and subsequent coupling with ammonium hydroxide. Melting points were determined by placing a small amount of the derivative into capillary tubes with one end sealed, and heating in n-butyl phthalate until melting occurred. Two melting point determinations were made for each derivative obtained.

The amino acid content of a combination of batches of crude toxin extract was determined with a Beckman amino acid analyzer, for comparison with studies which have reported high concentrations of aspartic acid (Lane, 1961), or glutamic acid (Lane and Dodge, 1958), or the absence of tyrosine (Phillips, 1956).

Crude toxin extracts were tested for carbohydrate materials by dissolving 100 mg of extract in 1 ml distilled water, and reacting with alpha-naphthol and sulfuric acid (Molisch test). Lipid content of crude toxin extracts was determined gravimetrically by extracting 100 mg samples with chloroform using the Sperry-Brand technique (Ackermann and Toro, 1953).

Reagent grade chemicals were used throughout this study.

RESULTS

Microscopic examination at intermediate stages in the preparation procedure revealed that the majority of nematocysts were of three types: basitrichous amastigophores, microbasic mastigophores, and microbasic amastigophores (terminology of Hand, 1961). Samples taken prior to incubation in distilled water had 1–10% of the nematocysts discharged. After incubation in distilled water samples were 72–92% discharged. Very little cell debris was noted in pre- or post-discharge samples. Batches of crude toxin extract were all toxic, although the degree of toxicity varied widely. No deaths occurred among control animals in any of the bioassays performed in this study, including those injected with sodium citrate at dose levels up to 750 mg/kg. Rehydration and incubation for 48 hours at 4° C was found to reduce the toxicity of crude toxin extract (relative potency less than

0.338). Heating for 30 minutes at 55° C also lowered the toxic activity of the extract (relative potency less than 0.093). Repeated lyophilization had no measurable effect on the activity of crude toxin extract.

Crude pellet extracts

Three batches of crude pellet extract were not lethal to any animals at dosages up to 750 mg/kg live body weight.

Results of dialysis

Calibration of dialysis tubing with various sugars revealed that the tubing used would pass material with molecular weight of 360 or less, but would not pass substances with molecular weight of 540 or more (a more precise estimate of the exclusion size of the tubing was not attempted, since this range would almost certainly prevent proteins from passing across the membrane).

TABLE I

Effect of dialysis and precipitation by barium hydroxide on nitrogen levels of material released by discharge of isolated nematocysts of Metridium senile

Sample Number	Total nitrogen (μg/mg extract)	Dialysable nitrogen (μg/mg extract)	Unprecipitable nitrogen (μg/mg extract)
1	2.00	0.70	0.74
2	4.20	2.00	2.40
3	1.90	0.90	0.92
4	0.90	0.24	0.29

Samples of crude toxin extract which had been dialyzed against a single 31 volume of distilled water were found to have increased protein concentrations of 18–34 fold. Bioassay, however, failed to demonstrate any increase in toxicity. Folin protein determination of these preparations revealed that about half of the material supposed to be protein in crude toxin extracts was dialyzable. Nitrogen determinations of dialyzable and undialyzable fractions showed that 30–50% of the nitrogen present in the crude toxin extract was dialyzable. In addition, the dialyzable nitrogen was found to correspond closely to the nitrogen remaining after crude toxin extracts had been deproteinized by barium hydroxide and zinc sulfate. These results are summarized in Table I. Crude toxin extracts, when deproteinized, still gave a blue color when treated with the Folin reagent. This reactivity disappeared, however, after dialysis.

Both dialyzable and undialyzable material were found to have toxic activity when assayed on *P. econfinae*, and in this case the undialyzable material had actually increased in toxicity relative to crude toxin extract assayed on the same species. The relative potency of the undialyzable material was 5.94, and the relative potency of the dialyzable material was 0.705.

Differential toxicity

As it seemed possible that *Procambarus econfinae* had a different sensitivity to the toxin preparations than *Orconectes propinquus*, the potency of crude toxin extract in *P. econfinae* was determined relative to *O. propinquus* and was found to be 2.56.

Fiddler crabs *Uca pugilator* and *Sesarma reticulatum* were injected with samples of extract at the LD_{50} level for *O. propinquus*. None of the animals in a 20-animal test group (each species) had shown any response (paralysis, autotomy, tetany or death) 24 hours after injection.

TABLE II

Amino acid analysis of material released on discharge of isolated nematocysts of Metridiun senile

Amino acid	Concentration in 1.00 g sample (μg)
Aspartic acid	204.022
Threonine	91.035
Serine	128.205
Glutamic acid	250.047
Proline	77.970
Glycine	103.500
Alanine	115.344
Valine	82.485
Methionine	41.571
Isoleucine	70.740
Leucine	114.363
Tyrosine	82.355
Phenylalanine	69.300
Total	1470.937
Unknown	approximately equimolar with proline

Results of paper chromatography

Paper chromatograms of crude toxin extract, dialyzable material, and undialyzable material all showed two spots with r.f. values of 0.20 and 0.42. The spots were less intense in the undialyzable fraction, but were distinct. In addition, a spot was observed at the point of application on chromatograms of crude and undialyzable material. This spot was ninhydrin positive, gave a blue Ehrlich reaction, and absorbed ultraviolet light. No further separation was obtained when two dimensional chromatograms were run. Both mobile spots gave a light blue fluorescence in ultraviolet light, a blue ninhydrin reaction, yellow Ehrlich reaction, reacted with copper sulfate, and gave a yellow-orange fluorescence in ultraviolet light when sprayed with potassium dichromate-40% formalin. The spot with r.f. = 0.20 produced a brown-orange spot when treated with Nessler's reagent. No reaction was observed with Dragendorf's reagent, 1-nitroso-2-naphthol, or acetylacetone-p-dimethylaminobenzaldehyde. Six batches of toxin run on the same chromatogram gave identical results.

Results of other characterization procedures

Ultraviolet absorption spectra show a slow decline from 200 mμ to 350 mμ without sharp peaks. This is probably due to inadequate purity of samples. Similar results were obtained for both crude and dialyzed materials.

Of the melting point derivatives obtained, only the picrate and benzenesulfonyl derivatives were useful. The other preparations decomposed in the region of 230° C without producing distinct melting points. The picrate derivative melted at 162–164° C, while the benzenesulfonyl derivative remained stable at temperatures up to 300° C.

Results of amino acid analysis on a combination of three batches are summarized in Table II. The quantities given were contained in 1.00 g crude toxin extract. The sum of these quantities is 1470 μg total amino acids. The amount of protein that should have been present in the analyzed material is 5392 μg, based on the Folin protein determinations of the original batches. If average concentrations (West and Todd, 1961, pages 280–281) are assumed for the amino acids not shown in the analysis, the quantity of amino acids is about one-third that of the Folin reactive material.

All batches of crude toxin extract were found to contain no carbohydrate material by the Molisch test, and only traces (less than 0.2%) of lipids by chloroform extraction.

Discussion

A significant quantity of the total nitrogen of nematocyst contents preparations was dialyzable. The dialyzable material reacts in a manner similar to that of protein when exposed to the Folin reagent. It is, however, very unlikely that the dialyzable substance is a protein because of the small particle size involved (molecular weight less than 540). The Folin method for protein estimation is based on reactions with tyrosine and peptide bonds. It seems probable that other aromatic amines might also react with the reagent, and this could account for the reduction of Folin reactive material in the dialyzed preparations. The amino acid analysis substantiates this explanation, because it indicates a protein level in crude toxin extracts which is about one-third of that indicated by the Folin method.

The possibility of the dialyzable substance being a small peptide was considered. Such a peptide would have to have a molecular weight of less than 540, which means that it could be composed of a maximum of seven amino acids (heptaglycine, mol. wt. 525). If a peptide such as this were present, it would react with the Folin reagent, and the component amino acids would be counted as part of the amino acid analysis of crude toxin extract. The total amino acids, however, accounted for only one-third of the Folin reactive material, and barely accounts for the total precipitable nitrogen. This makes the possibility of an unprecipitable, dialyzable amino acid compound unlikely, and indicates that there must have been present in the crude extract some substance other than amino acids capable of reacting with the Folin reagent.

The toxicity of crude and dialyzed preparation in *Procambarus econfinae* suggests that there are at least two toxic moieties, one dialyzable and the other undialyzable. If there are, in fact, two toxic fractions, there is a possibility of synergistic effects, such as those occurring in certain snake venoms (Dimitrov and Kankonkar, 1968). Recombining dialyzable and undialyzable materials failed to increase the activity, but this does not negate the possibility of synergism, since prolonged hydration was shown to cause some change in the toxic material. The fact that heating, as well as prolonged hydration, also abolishes toxic activity of the extracts, together with the amino acid analysis, indicates that the non-dialyzable fraction is proteinaceous.

The results of chromatography also indicate that a proteinaceous substance and dialyzable nitrogen compounds were present in the extracts. A non-mobile spot at the point of application on chromatograms of crude and undialyzable material is indicative of a protein-like substance in these materials. The reaction of the spot with ninhydrin and Ehrlich's reagent, and ultraviolet absorption are all characteristic of proteins. The two mobile spots were observed in crude, dialyzable, and undialyzable material. Their occurrence in the undialyzable fraction is not altogether surprising, since at equilibrium in the dialysis system there would theoretically be equal concentrations of dialyzable materials on both sides of the dialysis membrane, assuming no electrochemical effects occurred.

The blue ninhydrin reaction, combination with copper sulfate, and yellow Ehrlich reaction are all indicative of amines. Sky-blue fluorescence in ultraviolet light and reaction with potassium dichromate-40% formalin are indicative of aromatic amines. The reaction of one of the spots with Nessler's reagent indicates that this compound is carboxylated. It is unlikely that these compounds are indoles, serotonin analogs, or tetramethylated ammonium compounds, since no reaction was observed with reagents specific for these substances. Red colors with acetylacetone-p-dimethylaminobenzaldehyde were also not obtained, making the presence of hexosamines unlikely.

The formation of picrates is indicative of amines in general, while benzenesulfonyl compounds are formed by primary and secondary amines only. The melting points of these derivatives closely resemble the derivatives of certain intermediate compounds in tryptophan metabolism (*e.g.*, kynurenine, quinaldic acid), fused ring or polysubstituted aromatic compounds (*e.g.* naphthylamine) and histamine. The latter is noteworthy, in view of a suggestion by Welsh (1956) that, because of similarities in its pharmacology, histamine or a similar compound may be involved in toxic reactions to *Metridium* nematocysts.

Glutamic and aspartic acids were the most abundant amino acids in the hydrolysate of crude toxin extract. These findings are in agreement with those summarized by Picken and Skaer (1966).

It is interesting that many of the compounds which have been held by other workers to be responsible for nematocyst toxicity were not found in this study. Welsh and Prock (1958) reported a variety of quaternary ammonium compounds in extracts made from whole animals or tentacles of *Metridium dianthus.* Phillips (1956) reported the presence of serotonin in *Metridium senile* nematocysts, and Welsh (1960) found high concentrations of this substance in tentacles of the same species. Phillips and Abbott (1957) later noted, however, that the concentration of serotonin fell steadily as the toxin was purified (the nature of the purified toxin obtained by Phillips has not been published). Lentz and Wood (1964), using histochemical detection methods, have found serotonin both inside the nematocyst and the surrounding cnidoblast of *Metridium*. No indication was obtained in the present study of the presence of serotonin in either tentacles or crude toxin extract, nor was any indication found of the tetramethylated ammonium bases which have been reported.

The occurence of differential toxicity dependent upon the species of animal used for bioassay suggests that many seemingly contradictory studies may, in fact, be compatible. Welsh and Prock (1958, page 558) reported that extracts of *Metridium dianthus* tentacles contain "factors which, in considerable dilution, produce spontaneous autotomy of legs, followed by paralysis and death [in *Uca pugilator*]." Phillips and Abbott (1957) reported that extracts of isolated nematocysts were pharmacologically active in *Littorina planaxis*. In contrast, Mathias *et al.* found no pharmacologically active substance in macerated tentacles of *Metridium senile* when assayed in isolated organ preparations from a variety of vertebrates. In view of the highly variable response of different species of animals to the same extract in the present study, it is reasonable to suppose that the different findings actually reflect the difference in the assay organisms, rather than in the substances being assayed. Indeed, Welsh (1956) reported differences in pharmacological activity of tetramethylammonium, which mimics the action of *Metridium* toxins assayed on *Uca pugilator,* but has the opposite effect on *Hemigrapsus nudus*.

Although research on Scyphozoan nematocyst toxins has been comparatively scanty the available information suggests that there are similarities between Scyphozoan and Anthozoan toxins. Welsh (1956) found evidence of an amine in *Cyanea* tentacle extracts which has excitory action when assayed on the isolated heart of the horse clam *Schizothaerus nuttallii*. In a later paper, Welsh and Prock (1958) reported the presence of tetramethylammonium, homarine, trigonelline, and γ-butyrobetaine in tentacles of *Cyanea*, but pure samples of these compounds did not have the same pharmacological activity as *Cyanea* tentacle extracts when assayed on *Uca pugilator*. Barnes (1966) has reported on the occurrence and pharmacology of the Irukandji carybdeid, *Chironex fleckeri,* and *Chiropsalmus quadrigatus* which are a serious health hazard in tropical waters. The symptoms produced are skin wheals, edema, deep tissue necrosis, and cardiac arrest. The lack of paralysis and general organ pathology suggest that the toxic agent(s) may be similar to those found among the Anthozoa.

The obvious discrepancies in reported toxin compositions among the various studies are probably due to tissue contaminants in various preparations. The little work which has been done with isolated nematocysts seems to indicate that the toxin of a particular species of cnidarian is fundamentally simple, and there is, in fact no direct link between any of the substances reported in tissue extracts and the toxicity of nematocyst contents. The results of this study indicate that the toxin of *Metridium senile* is also simple, in that it may be composed of a protein and one or two aromatic amines, which previously have not been reported.

SUMMARY

1. Material discharged by isolated nematocysts of *Metridium senile senile* Linnaeus (1767) has been found to be toxic to crayfish *Orconectes virilis* Hagen (1870), *O. propinquus* Girard (1852), and *Procambarus econfinae* Hobbs (1942).

2. The toxin appears to be composed of a proteinaceous, undialyzable fraction, and a dialyzable fraction containing two aromatic amines.

3. The amines are probably fused or polysubstituted ring compounds, one of which is carboxylated.

4. The crude toxin has varying potency, depending upon the species of assay animal used.

5. With the possible exception of histamine, none of the compounds previously suggested as possible components of the nematocyst toxin were found.

LITERATURE CITED

Ackerman, P. G., and G. Toro, 1963. Blood lipids. Pages 249–272 in S. Frankel, S. Reitman, and A. C. Sonnenwirth, Eds., *Clinical Laboratory Methods and Diagnosis.* C. V. Mosby Co., St. Louis.

Barnes, J. H., 1966. Studies on three venomous Cubomedusae. Pages 307–332 in W. J. Ross, Ed., *The Cnidaria and their Evolution.* Academic Press, Aberdeen.

Blanquet, R., 1968. Properties and composition of the nematocyst toxin of the sea anemone *Aiptasia pallida. Comp. Biochem. Physiol.*, **25**: 893–902.

Bliss, C. I., 1952. *The Statistics of Bioassay.* Academic Press, New York, 183 pp.

Bregoff, H. M., E. Roberts and C. C. Delwiche, 1953. Paper chromatography of quaternary ammonium bases and related compounds. *J. Biol. Chem.*, **205**: 565–574.

Dawson, R. M. C., D. C. Elliot, W. H. Elliot and K. M. Jones, 1959. *Data for Biochemical Research.* Clarendon Press, Oxford, 299 pp.

Dimitrov, G. D., and R. C. Kankonkar, 1968. Fractionation of *Vipera ruselli* venom by gel filtration. II. Comparative study of yellow and white venoms of *Vipera russelli* with special reference to the local necrotizing and lethal actions. *Toxicon*, **5**: 283–288.

Hand, C., 1954. The sea anemones of central California. *Wasmann J. Biol.*, **12**: 3–251.

Hastings, S. G., J. B. Larsen and C. E. Lane, 1967. Effects of nematocyst toxin of *Physalia physalis* on the canine cardiovascular system. *Proc. Soc. Exp. Biol. Med.*, **125**: 41–45.

Kline, E. S., and V. S. Waravdekar, 1960. Inhibitor of succinoxidase activity from *Hydra littoralis. J. Biol. Chem.*, **235**: 1803–1808.

Lane, C. E., 1961. *Physalia* nematocysts and their toxin. Pages 169–178 in H. M. Lenhoff and W. F. Loomis, Eds., *The Biology of Hydra.* University of Miami Press, Coral Gables, Florida.

Lane, C. E., and E. Dodge, 1958. The toxicity of *Physalia* nematocysts. *Biol. Bull.*, **115**: 219–226.

Lentz, T. L., and J. G. Wood, 1964. Amines in the nervous system of coelenterates. *J. Histochem. Cytochem.*, **12**: 37.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

Martin, E. J., 1966. The macromolecular toxin of sea anemones. Pages 136–138 in R. I. Bowman, Ed., *Proceedings of the Symposia of the Galapagos International Scientific Project, 7–18 Jan., 1964, Berkeley.* University of California Press, Berkeley.

Mathias, A. P., D. M. Ross and M. Schacter, 1960. The distribution of 5-hydroxytryptamine, tetramethylammonium, homarine, and other substances in sea anemones. *J. Physiol.*, **151**: 296–331.

Meites, S., and W. R. Faulkner, 1962. *Manual of Practical Micro and General Procedures in Clinical Chemistry.* Charles C Thomas, Springfield.

Phillips, J. H., 1956. Isolation of active nematocytes of *Metridium senile* and their chemical composition. *Nature*, **178**: 932.

Phillips, J. H., and D. P. Abbott, 1958. Isolation and assay of the nematocyst toxin of *Metridium senile fimbriatum. Biol. Bull.*, **113**: 296–301.

Picken, L. E. R., and R. J. Skaer, 1966. A review of researches on nematocysts. Pages 19–50 in W. J. Ross, Ed., *The Cnidaria and their Evolution.* Academic Press, Aberdeen.

Richet, C., and P. Portier, 1936. Recherches sur la toxine des coelenteres et las phenomenes d'anaphylaxie. *Resultats des campagnes scientifiques Monaco,* **95**: 3–24.

Snedecor, G. W., and W. G. Cochran, 1967. *Statistical Methods.* Iowa State University Press, Ames, Iowa, 593 pp.

Udenfriend, S., C. T. Clark and A. Neissbach, 1955. The estimation of 5-hydroxytryptamine in biological tissues. *J. Biol. Chem.,* **215**: 337–344.

Welsh, J. H., 1956. On the nature and action of coelenterate toxins. *Deep Sea Res. Supp.,* **3**: 287–297.

Welsh, J. H., 1960. 5-hydroxytryptamine in coelenterates. *Nature,* **186**: 811–812.

Welsh, J. H., 1961. Compounds of pharmacological interest in coelenterates. Pages 179–186 in H. M. Lenhoff and W. F. Loomis, Eds., *The Biology of Hydra.* University of Miami Press, Coral Gables, Florida.

Welsh, J. H., and P. B. Brock, 1958. Quaternary ammonium bases in the coelenterates. *Biol. Bull.,* **115**: 551–561.

West, E. S., and W. R. Todd, 1962. *Textbook of Biochemistry.* Macmillan Company, New York, 1356 pp.

Yanagita, T. M., 1959. Physiological mechanism of nematocyst responses in sea anemone. *J. Exp. Biol.,* **36**: 478–494.

Reference: *Biol. Bull.*, **140**: 400–415. (June, 1971)

TEMPORAL PATTERNS OF SEXUAL AND ASEXUAL REPRODUCTION IN THE COLONIAL ASCIDIAN *METANDROCARPA TAYLORI* HUNTSMAN[1]

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Metandrocarpa taylori is a colonial ascidian common on rocks in the low intertidal zone along California shores. Reddish-orange colonies spread out in flat sheets, often covering large expanses of granite rocks, particularly on their lower surfaces. Individuals, which resemble small simple ascidians, are joined to one another basally by a thin layer of test containing blood vessels.

Previous studies on *M. taylori* have been concerned largely with bud development (Abbott, 1953), analysis of the factors involved in bud initiation (Newberry, 1965), and larval structure and behaviour (Abbott, 1955).

The major objective of the present work is the comparison of sexual and asexual reproductive activities in *M. taylori*, including consideration of how these two processes interact in colonies throughout the year. There is also a brief consideration of the seasonal aspects of reproduction in relation to water temperature and phytoplankton. A study of the gonads was also necessary in order to determine criteria for sexual reproductive activity. Except for the observation of Abbott (1955) that larvae settled in the summer months, nothing has been recorded as to the extent of either the sexual or asexual reproductive cycle. Likewise, there is no adequate description of the gonads in the literature; Huntsman (1912) and Ritter and Forsyth (1917) described populations of *M. taylori* that differed somewhat from each other in the number and arrangement of the gonads.

Materials and Methods

Specimens of *Metandrocarpa taylori* used in the study of sexual reproduction were collected at Pescadero Point, Monterey County, California (36°31′47″N.) at approximately two week intervals from June 1, 1961 to June 2, 1962. Colonies were sampled by using a chisel to remove clusters containing about a dozen individuals each. Approximately ten different colonies were sampled on each collecting date. The specimens were relaxed for 12–24 hours in a 7% $MgSO_4$-seawater solution, then fixed in Bouin's fluid and stored in 70% EtOH. Adults in each cluster, connected basally by a common tunic, are readily separated from one another intact. Gonads are easily exposed by removing the upper half of the zooid by a horizontal cut, then carefully lifting out the remaining portion of the branchial basket along with the stomach and intestine.

Since *M. taylori* usually occurs on irregular surfaces in situations where growth is difficult to monitor, colonies used in the study of asexual reproduction were established on glazed tiles and maintained in racks attached in the intertidal zone adja-

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cent to Hopkins Marine Station. Three clusters of adult zooids from separate colonies were collected each month at Pescadero Point, cleaned of debris, and tied with thread to the center of six-inch square glazed ceramic tiles. Tiles were kept in running seawater for a month in the lab, to allow the zooids to attach and begin to bud. The tiles were then placed in slotted redwood boxes bolted to intertidal rocks protected from heavy surf approximately 0.2 feet above mean lower low water.

Once a month, all of the tiles bearing cultures were transferred to laboratory aquaria, observed, photographed with a Polaroid camera, and returned to the sea boxes within 24 hours. Individual colonies were maintained until they grew to the edges of a tile. While there was some damage and loss of colonies due to storms, most colonies expanded to cover one side of the tile by the end of four months in the intertidal zone.

The gonads of twenty adult zooids collected biweekly from different colonies were measured to provide data on sexual reproduction. Female gonads were assigned to the following size classes: small (ovaries less than 0.1 mm in diameter); medium (ovaries 0.1 mm or more in diameter, eggs still encased in ovarian epithelium); and developing (mature eggs and various stages of embryos, both lying free in the mantle cavity). Size classes for testes were small (less than 0.2 mm in greatest diameter); medium (0.2–0.4 mm), and large (over 0.4 mm).

Results

Gonads

In *Metandrocarpa taylori* the separate testes and ovaries are partially embedded in the ventral floor of the mantle cavity. The ovaries extend in a horseshoe shape, back and outward on both sides of the medial endostylar ridge. The testes extend posteriorly from these on each side, in a line on the right but bunched on the left due to presence of the stomach. The gonads are illustrated in Haven (1965). The average number of ovaries was seven (range 0–13) and the average number of testes was nine (range 0–17). The number and the arrangement of gonads in *Metandrocarpa taylori* are in general accord with the reproductive system described by Ritter and Forsyth (1917) for *M. michaelseni*, a species placed in synonomy with *M. taylori* by Abbott (1953).

In animals not actively engaged in sexual reproduction the ovaries are small (less than 0.1 mm in diameter) and contain developing eggs in early stages. Three or four enlarging eggs lie in each ovary. As the eggs enlarge and become yolky, they stretch the overlying epithelium. No more than one or two eggs are fully mature at the same time in an individual ovary and it appears that usually only one egg is released at a time from an ovary.

When the eggs reach a diameter of approximately 0.3 mm they are released from the ovary, apparently by rupture of the gonad epithelium since an oviduct is lacking, and come to lie free in the mantle cavity. Fertilization probably occurs at this time. The larvae are usually retained in the adult until they are capable of swimming away to settle (Abbott, 1955).

Small testes (less than 0.2 mm in diameter) have a rounded base and a vas deferens protrudes from the center of each. Sections of testes of this size show densely packed cells with no lumen evident. Testes of intermediate sizes (0.2 mm–0.4 mm in diameter) are pear-shaped. Smears of these testes show many nurse cells with attached spermatids, together with immature sperm. With further enlargement (over 0.4 mm diameter) the testes develop lobulations, and smears contain a good proportion of highly motile sperm. Collections frequently contained a number of adult zooids with no visible testes, suggesting the possibility of complete regression of the testes during periods of inactivity.

Sexual reproduction in the population

The annual pattern of sexual reproductive activity, based on the most advanced stages of gonads present in individuals, is shown in Figure 1. Sexual reproduction continues throughout the year. At least 10% of the population always showed some developing embryos and larvae, and these stages were present in a maximum of 70% of the sample in the collection of July 27, 1961. In addition, medium sized eggs were present in other individuals from 5% to 50% of the time. At no time was less than 30% of the population engaged in sexual reproduction, as indicated by the presence of medium sized eggs and/or developing embryos and larvae. Summer was the period of greatest sexual activity. In the three month period from June 12, 1961 to September 11, 1961, an average of 79% of the population contained medium sized eggs or developing larvae or both and the number of individuals containing developing embryos and larvae was 30% higher than in the remainder of the year.

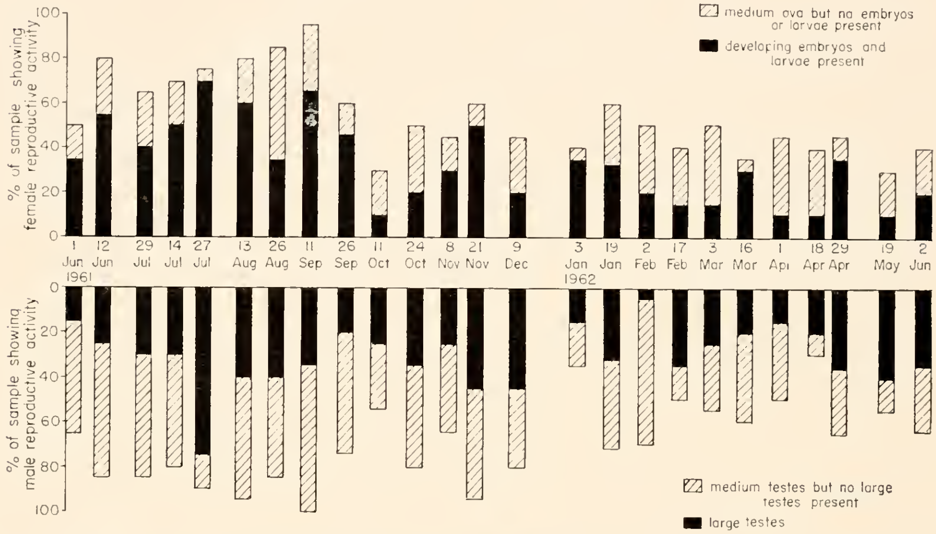

FIGURE 1. Sexual reproductive activity of *Metandrocarpa taylori* in the collections of 1961–1962. Zooids are classed according to the most advanced condition of reproductive activity of the male and female systems present. Dates of collection are shown on the horizontal scale.

The lower half of Figure 1 shows the condition of the testes in each collection. During the summer, when embryo production was increased, there was no corresponding increase in the proportion of individuals with large testes, but increased summer activity is indicated when the classes with medium sized and large sized testes are considered together. Throughout the year a rise or fall in the incidence of medium and large testes usually corresponded to a similar change in the incidence of medium sized and developing eggs; the correlation coefficient for this was highly significant ($r = 0.784$; $P < 0.0005$).

Collections of *M. taylori* taken by Dr. Donald P. Abbott of Stanford University from April 1949 to March 1950 were similarly analysed and also showed greater sexual reproduction during the summer months and early fall.

TABLE 1

Summary of egg and testes stages present simultaneously in individual zooids

Individual Zooids with:	Collections of 1949–50 period (N = 440)	Collections of 1961–62 period (N = 540)
No ovary visible	13%	1%
Small eggs only	43	45
Small + medium sized eggs	11	20
Small, medium sized and developing eggs	9	13
Medium sized + developing eggs	15	16
Developing eggs only	4	2
Small + developing eggs	3	2
Medium sized eggs only	2	1
Total	100%	100%
No testes visible	10%	10%
Small testes only	22	21
Small + medium testes	10	3
Medium testes only	30	35
Medium + large testes	11	8
Large testes only	17	23
Total	100%	100%

Goodbody (1961) has questioned the measurement of gonad conditions in ascidians as an accurate measure of sexual activity, citing resorption of unfertilized winter eggs in *Botryllus schlosseri*. However, larvae of *M. taylori* settled throughout the year on glass slides kept in the intertidal zone at Hopkins Marine Station by Dr. A. T. Newberry, University of California, Santa Cruz (personal communication). Therefore, dormancy or arrested development is unlikely in the present case. Also, the peak occurrences during the year of individuals just completing a phase of sexual reproduction (*i.e.* having medium and developing but no small eggs) corresponds to peaks of individuals entering such a phase (having small and medium eggs only) three weeks previously. This suggests that eggs are not held in a dormant state, but become developing embryos and larvae in a predictable period of time. The fluctuations in the occurrence of zooids entering a period of sexual reproduction (those having small and medium eggs) lend support to the reality of the seasonal variations in sexual reproduction shown in Figure 1.

A remarkably large reservoir of small eggs is present in the population throughout the year. This reserve is quite constant and is only slightly depleted during periods of increased sexual reproduction. This is partly because a zooid appears to replace almost immediately any small eggs that develop during a period of active sexual reproduction.

The portion of the population containing at least some small testes is relatively low most of the year. Individuals with medium sized testes form the most frequent male component in the population. It is possible that less time is required for a small egg to enlarge and be released from an ovary than for a small testis to enlarge and produce ripe sperm. If this is the case, the more immediately available reserve of male gonads would necessarily be the individuals with medium sized testes.

The reproductive condition in individual zooids

Because the adult zooid is opaque, the sequence of reproductive events cannot be determined directly by repeated observations on particular individuals. However, since individual zooids may contain simultaneously eggs, testes, and embryos in several different stages of growth, inferences can be made about the sequence of gonad development, including the relationship of male to female gonad development within individuals.

Analysis was made of egg stages present simultaneously in 980 individual zooids (Table I). When a zooid is reproductively active, the eggs within are usually in more than one class and these classes represent developmental stages that are in sequence with one another. The data suggest the following sequence of egg development within the zooid. When a zooid is not reproductively active, each ovary contains several small eggs. With the onset of reproduction, these eggs enlarge; individuals containing both small and medium sized eggs represent this point in the life history of a zooid. Later, all three stages of eggs can be found, and finally all the small eggs enlarge and individuals contain only medium sized and developing eggs. However, before this batch of eggs is entirely released, small eggs again appear in the ovaries. The data cannot show whether individual zooids ripen eggs more than once during a period of active reproduction of the colony as a whole.

The size classes of testes present simultaneously in individuals are summarized in Table I. In 85% of all the individuals examined, the testes fall into one size class. In the remainder of specimens, where more than one size class of testes was present, the size classes were developmentally adjacent. This indicates that all the testes within an individual usually enlarge simultaneously, or at least in relatively close synchrony with one another.

Within individual zooids the development of testes and ovaries is positively correlated. Most of the time, when eggs are small or ovaries are not visible, the testes are small or not visible grossly. When eggs have begun to enlarge in the individual, the testes are usually medium and/or large. The per cent of individuals with large testes increased with the presence of developing eggs, and reaches a peak in zooids containing only medium sized and developing eggs.

Whether or not self-fertilization occurs in *M. taylori* is unknown, but since the colonies appear to be clones that are established asexually from a single metamorphosed larva, cross-fertilization within the colony would be equivalent to self-fertilization.

Temporal patterns of asexual reproduction

Metandrocarpa taylori is a colonial tunicate, of the "social" type, with zooids connected basally, but each individual having a separate covering of test. Colonies are produced asexually by pallial budding along the free basal margins of the mantle (for details of bud growth and differentiation, see Abbott, 1953). Throughout its life the newly budded zooid maintains contact with its parent and with its own subsequent bud progeny by means of blood vessels traversing the tunic-covered connections between them. Buds are projected far enough from the parents so that there is room for eventual enlargement to adult size. In a colony growing on a clean, unobstructed surface an additional distance of approximately two millimeters separates young zooids. This allows large areas to be fairly rapidly colonized, the spaces within the colony being filled later by intercalary budding.

Zooids used to establish experimental colonies were taken from mature colonies in the field and were closely packed, with basal margins fused throughout. These zooids were not actively budding at the time of collection, since new buds arise only from areas on zooids where the basal test margin is free, and not fused to that of adjacent zooids (Abbott, 1953). When the test margins were freed at the edges of the clumps of adults used to establish new colonies on tiles, most of the clusters began to produce buds. The ability to produce buds appears to be potentially present in zooids of *M. taylori* throughout life.

New colonies were established on tiles each month for a year, to provide a continuous and overlapping record of asexual reproductive activity. Figure 2 shows the form and history of a typical colony cultured in the intertidal zone for four months. The adults which founded the colony are centrally located. New growth radiated outward from these original zooids as buds appeared on the periphery. As the colony grew larger, the older zooids toward the center of the colony also enlarged, and their test margins coalesced, with some intercalary budding filling most of the remaining space. Thus the center of the colony became more and more solidly packed with zooids and produced no more buds after reaching this packed condition.

Photographs taken each month of each colony cultured on a tile provided a series of growth records extending throughout a year. As the first step in translation of these records into statistical data, areas of new growth each month were outlined and mapped in diagrammatic tracings made from the original photographs. The vascular connections retained between each zooid and its parent allowed the determination of the lineage of all new buds grown during a month. A bud was counted as a new zooid only when its budstalk attachment to its parent was clearly broken. Once the growth was outlined, the zooids were classified as to their order of descent. In Figure 2, zooids enclosed within a heavy line are part of the base population present at the end of the previous month. Zooids giving rise directly to new growth, lying outside of a heavy line, are termed "budding adults." Buds ("descendents") produced during the month are assigned generation numbers in relation to the budding adult of the previous month from which they stem; those buds arising from a budding adult are considered "first generation," and all of the buds traceable to them during a month are on a single "clonal line."

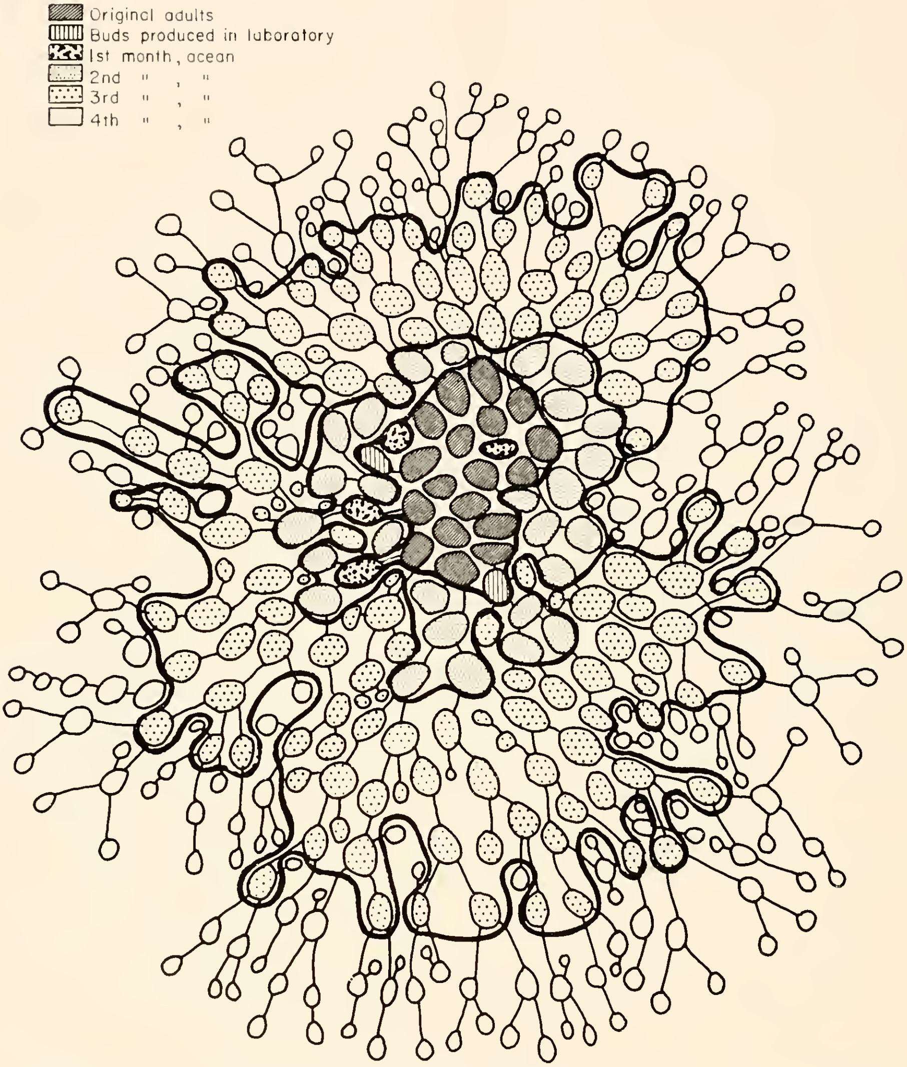

FIGURE 2. Budding pattern of a colony of *Metandrocarpa taylori* cultured on a glazed ceramic tile for four months in the intertidal zone. Growth during each successive month is circumscribed by a darkened contour line.

In measuring growth rate, only budding rate expressed as average number of descendents produced per budding adult during the month expressed growth in a meaningful way. This was calculated by dividing the total number of new individuals produced during the month by the number of budding adults giving rise to them; the final calculation expressed as number of descendents per budding adult per day.

Effects of colony size and age

Two aspects of a colony, its size and its age, might be expected to affect budding rates. With respect to size, the adults in the interior of a colony usually have no room to bud but they might provide an additional source of nutrition to budding zooids at the colony margin through the anastamosing vascular network of the colony. If this were the case, a large colony, with a high percentage of non-budding adults, might be expected to bud at a faster rate than smaller colonies. Data from all months were pooled to see if any correlation existed between budding rate and colony size. There was none. Of course, the *number* of adults that do bud and produce a month's growth is clearly related to the size of a colony. This positive correlation is due to the expansion of the periphery of the colony (where most budding adults occur) as the total size (area) of the colony increases.

With regard to the effect of age on budding rate, determinations of average growth rate for any particular month of the year were based on growth occurring in colonies on tiles that had been in the ocean for differing amounts of time. Neither the zooids nor colonies as whole units appear to have a fixed life span, as do some tunicates; while adult zooids do die from time to time, a colony of *Metandrocarpa taylori* does not undergo periodic fluctuations in population due to death of adult zooids. The possibility remains, however, that there may be other effects of growth rate due to the age of a colony.

Data on the budding activity for colonies that had grown for similar lengths of time in the ocean (regardless of season), showed that the budding rate of colonies during their first month in the intertidal zone (0.77 buds/budding adult/day) was significantly lower than that found in any of the succeeding months. This lowered rate of growth probably represents a period of acclimation, since the colonies had previously been in laboratory tanks for a month. Since this rate does not reflect any conditions of growth that might be encountered in field populations of *M. taylori*, seasonal budding rates in all instances have been based on growth occurring in colonies *after* this first month in the intertidal zone.

Budding rates in colonies during their second, third, and fourth months in the ocean were 0.122, 0.125, and 0.135 buds/budding adult/day, respectively. An analysis of variance showed that the differences between these rates were not significant ($F_{2,45} = 0.245$). Only one colony provided data on growth during the fifth month in the ocean (0.111 buds/budding adult/day).

Annual pattern of asexual reproduction

Asexual reproduction is exhibited throughout the entire year in colonies of *Metandrocarpa taylori* cultured in the intertidal zone (Fig. 3A). There is, however, marked variation in average monthly budding rates during this period. Budding activity was high in June 1961, but declined rapidly thereafter and continued at a slow rate for the rest of the summer, coming virtually to a standstill in September. A burst of asexual reproductive activity followed in October and budding rates remained high during the remainder of the year under study with

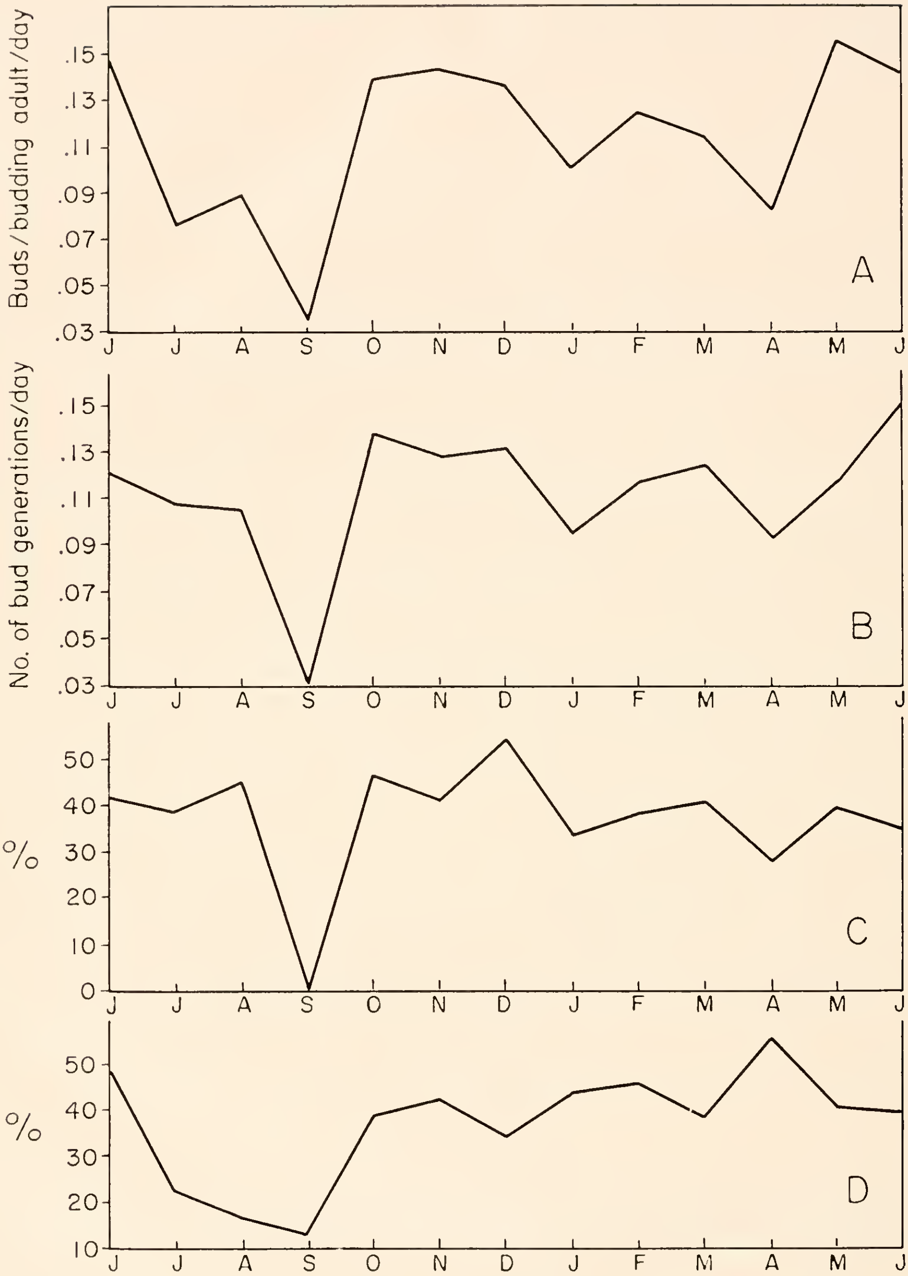

FIGURE 3. Comparative activity of components determining budding rate; (A.) average monthly budding rate in colonies of *M. taylori;* (B.) average of maximum number of bud generations produced per day; (C.) average per cent of zooids produced during a month that in turn produced buds; (D.) incidence of multiple budding: average per cent of all zooids producing buds during a month that gave rise directly to more than one bud.

the exception of a drop in budding rates during April, 1962. Budding activity at this time was lowered to a rate comparable to that evidenced in the previous August. An apparent drop-off in budding rates occurred during January. In this case, however, growth rate is based on measurements in a single colony, and the drop is of dubious significance.

Components of growth rate change within a colony

An analysis of budding activity in *Metandrocarpa taylori* shows that there are three ways in which a colony may vary its rate of bud production in a given period of time: (1) the rate at which successive bud generations follow one another may vary, so there is a change in the number of bud *generations* produced per unit of time. (2) The average number of bud offspring produced directly by those zooids which bud may vary with the season; *i.e.,* during certain periods the incidence of twin and triplet bud offspring may be greater than at other times. (3) The number of terminal budding adults which fail to bud at all may vary with the season. An increase or decrease in any one of these would affect the total rate of bud production. If all of these methods were more or less equally involved in producing changes in budding rate, asexual reproduction might then be considered directly related to the overall metabolism of a colony (interactions with sexual activity being ignored for the present analysis). If, however, the relative roles of each of these factors involved in budding rates shifts from time to time, a more complex picture of the components involved in colony growth emerges. An analysis of each of the factors follows.

(1.) *Maximum number of generations produced per unit time.* The maximum rate of production of new zooid generations each month (Fig. 3B) varies in parallel with overall colony growth rate throughout the year (Fig. 3A). Bud generation time varies inversely with colony growth rate. For example, the bud production rate for September of one generation in thirty days contrasts with a rate of one generation every six days in October.

(2.) *Incidence of multiple budding.* All zooids that are not terminal on a clonal line have given rise to at least one bud. The spreading pattern of growth encountered in colonies is accomplished by the production of two or more buds on the part of at least some of the parental zooids. Occasionally, as many as six buds have been found coming from a single zooid. The zooids that produced buds during a given month (including the budding adults that were parental to the first generation of buds) were divided into two categories: those zooids that produced only one bud, and those that produced two or more buds. The seasonal trends in overall budding activity and the incidence of multiple budding (Fig. 3D) are similar (low in the summer and high in the winter). However, fluctuations within the seasons show little relation between the two rates.

(3.) *Per cent of buds becoming parental.* During the year under study, an average of 40% of zooids that were produced each month in turn produced buds during that month. However, the variation found ranged from an absolute minimum in September, to a high point in December, when 55% of the zooids produced during that month gave off buds. When this rate is compared with the annual budding rate (Fig. 3C) there are similarities in the direction of change (*i.e.,* increase or decrease), but the amount of this change for corresponding periods in the two curves sometimes differs widely (*e.g.*, June–July, 1961; April–May, 1962).

The effect of changes of activity in the three components of budding on overall budding rate is more clearly observed in those months in which budding rate rose or fell markedly in relation to the previous month. Major changes in budding rate in colonies of *Metandrocarpa taylori* frequently involve parallel shifts in all of the three growth components enumerated. However, during three of the months, budding rate changes are accounted for by shifts in the number of generations produced and the number of new buds that became parental, but not in the amount of multiple budding taking place.

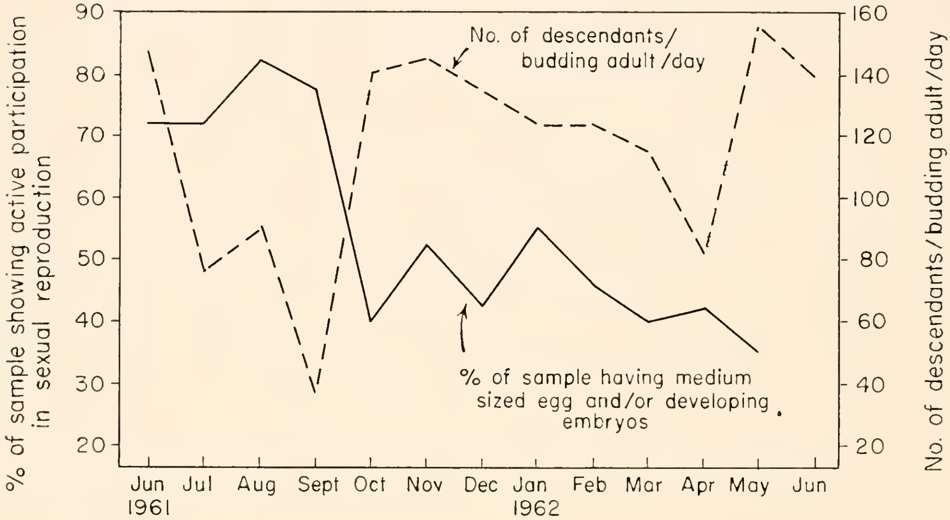

FIGURE 4. Monthly average buding rate in cultured colonies of *M. taylori* during 1961–62 compared with sexual reproductive activity in field populations during the same period.

Comparison of sexual and asexual reproductive activity

Both sexual and asexual reproduction occur throughout the year in *M. taylori.* The seasonal interrelationships of the two modes of propagation are outlined in Figure 4. In late summer and early fall, sexual reproduction is at its highest level, while asexual reproduction is declining toward its minimum. For the remainder of the year, while sexual reproduction is fluctuating at a relatively low level, asexual reproduction is, for the most part, at a relatively high level. The most striking changeover occurs during the sudden steep decline of sexual reproduction in late September–early October. During the same period, there is an equally large and sharp increase in asexual reproduction.

Correlation of reproductive patterns with temperature and food

Giese (1959) notes that reproductive cycles may be the result of endogenous factors, exogenous factors, or a combination of both. Nothing is known of endogenous factors in *Metandrocarpa taylori* that might account for cyclic reproduction. The exogenous factors often considered to influence sexual reproduction in marine organisms are cited by Giese: light, salinity, temperature and food. Salinity changes very little during the year in Monterey Bay, the average annual range (based on a seven-year period) representing a variation of less than one part per thousand (Bolin and Abbott, 1963). The other two factors are considered here. Phytoplankton records are based on plankton hauls averaged for six stations in Monterey Bay taken each week by the Hopkins Marine Station vessel TAGE, working under the auspices of California Cooperative Oceanic Fisheries Investigations program.

In 1961–62 there is a general direct relationship between sexual reproductive activity in *M. taylori* and ocean temperatures (Fig. 5). In summer and early fall, when temperatures were high, sexual reproduction was also high, reaching a peak in September when 96% of the sample showed reproductive activity. During the winter and spring, when water temperatures were low, sexual activity fluctuated about a relatively low mean value of about 45%. The correlation coefficient (r) for temperature and sexual reproductive activity (per cent of sample containing medium and/or developing eggs) was positive and highly significant: $r = 0.616$, $P < 0.005$. The correspondence between temperature and sexual reproductive trends, however, is not exact enough to allow the conclusion that temperature is the only important controlling factor. Only half of the time were rising water temperatures accompanied by increased sexual reproduction during 1961–62, and a drop in average ocean temperature was mirrored by a decrease in sexual activity in only one-third of the cases. In particular, there was no abrupt change in temperature with which the very sharp drop in sexual activity occurring in late September could be correlated.

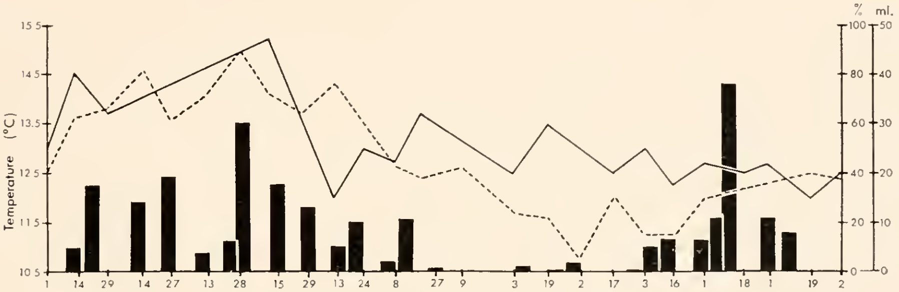

FIGURE 5. Seasonal changes in temperature and phytoplankton crop in relation to sexual reproduction in *M. taylori*. Solid line indicates per cent of sample containing medium sized eggs and/or developing embryos and larvae. Dashed line shows daily shore temperatures taken at Hopkins Marine Station averaged for period preceding each collection. Bars indicate average phytoplankton volumes (milliliter wet settled phytoplankton/haul) in Monterey Bay.

When budding activity in the cultured colonies was compared with the average shore temperatures for the same period, there was a general inverse relationship between the two. However, temperatures and asexual reproduction showed little detailed correspondence throughout the year, and this suggests that the effects of temperature, if any, are indirect and asexual reproductive activity is influenced by other factors.

Phytoplankton crops along the central California coast are highest during the summer months, a time when sexual reproduction is particularly marked in populations of *M. taylori* (Fig. 5). Summer phytoplankton volumes reached a peak in early September in 1961 and sexual reproductive activity was highest soon thereafter. However, the relationship between phytoplankton crop and amount of sexual reproduction does not appear as direct when conditions during the winter and spring months are taken into consideration. For example, sexual activity rose late in January without any apparent change in minimal phytoplankton levels. Conversely, a marked bloom of phytoplankton in mid-April was not accompanied or immediately followed by a rise in sexual reproductive activity.

When budding activity was compared with phytoplankton volumes there was no direct relationship found.

Discussion

The finding that the colonies of *Metandrocarpa taylori* reproduce sexually throughout the entire years is not totally unexpected considering that the eggs are yolky, and the larvae are brooded until fully developed. Furthermore, the larvae are wholly lecithotrophic, and more than 50% of them settle within two hours after release from the parent (Abbott, 1955). In invertebrates breeding in such a manner, with the larvae undergoing nonpelagic development, species are likely to exhibit a prolonged breeding season (Giese, 1959) and may spawn during the whole year (Thorson, 1946).

Although phytoplankton has seasonal blooms, there is an abundant food supply available to *M. taylori* during the entire year. Glynn (1965) made periodic samples of water washing over the intertidal zone during high tide in the Monterey Bay area. The results showed that there is a good supply of organic detritus suspended in shore waters throughout the year. This material is particularly abundant during the winter and spring months, a time when phytoplankton crops are minimal; in this period organic materials, such as kelp and other seaweeds, are torn loose and churned up by storm waters (Boolootian, Giese, Farmanfarmaian and Tucker, 1959). MacGinitie (1937) points out that an abundance of food may be available to ciliary-mucoid feeders, since even bacteria can be successfully captured. *Metandrocarpa taylori,* a ciliary-mucoid feeder, has available to it phytoplankton, bacteria and organic detritus that is suspended in inshore waters.

Sometimes simultaneous sexual and asexual reproduction may occur in animals with a life span shorter than a year (Berrill, 1951). This serves to heighten the interest in what occurs in *M. taylori,* in which the colony has no known definite life span and survives longer than annual forms. Asexual reproduction in *M. taylori* represents a type of growth, and this species falls in the general category of organisms, recognized by Orton (1920), in which major periods of growth occur in the non-breeding season.

The lower intertidal zone where *Metandrocarpa taylori* is found in the field is replete with invertebrates, and the rocks are seldom bare except when tossed about and scoured during winter storms. Winter turbulence could make it more difficult for larvae of *M. taylori* to settle. Also, the impact and the scouring action of waves might reduce the chances of survival of juvenile animals on the rocks during the winter. If this is true, there may be a mild selective pressure toward reduction of sexual reproduction during the winter. Budding would certainly be advantageous during this time as a means of recouping losses to colonies due to scouring action and increasing areas colonized. This may in part explain the selective value of continuous reproduction in *Metandrocarpa taylori,* with alternate stress on sexual and asexual methods.

In other styelid ascidians studied, seasonal breeding has been commonly noted; *Pelonaia corrugata,* deep off the Scottish coast breeds for only two to four weeks in January or February (Millar, 1954a) and *Styela rustica,* off the coast of Sweden has a similarly abbreviated breeding period during January and February (Lützen, 1960). On the other hand, longer periods of reproduction, extending roughly from spring to fall, are found in *Styela coriacea* off the northern German coast (Diehl, 1957), and *Dendrodoa grossularia* in shallow Scottish coastal waters (Millar, 1954b). *Styela plicata* has both a spring and a fall breeding season, where it occurs in the Lagoon of Venice (Sabbadin, 1957). In contrast to the breeding seasons noted above, *Symplegma viride,* a tropical-subtropical species and the only styelid tunicate among those listed here which reproduces both sexually and asexually, breeds throughout the year on the coast of Jamaica (Goodbody, 1961).

Reproductive cycles in two other ascidians of the Monterey area have been recorded. Trason (1963) found reproduction in *Pycnoclavella stanleyi* similar to that noted in the present study; both sexual and asexual reproduction occur throughout the year, with sexual reproduction occurring more actively in summer months. Levine (1962) reports cyclic sexual reproduction in *Eudistoma ritteri,* with larvae produced from mid-April to September.

Three invertebrates, other than ascidians, have been found that breed continuously and with little apparent fluctuation in the Monterey area; these are the red abalone, *Haliotis rufescens* (*cf.* Boolootian, Farmanfarmaian, and Giese, 1962), the kelp crab, *Pugettia producta,* and the porcelain crab, *Petrolisthes cinctipes* (*cf.* Boolootian *et al.*, 1959). These findings correspond to Giese's observation (1959) that, although a restricted period of breeding seems to be the general case in marine invertebrates, most invertebrates in temperate seas spawn over a much longer period of time than do those living in polar regions, and in a few temperate region species, spawning may last for the greater part of the year.

Most marine animals investigated thus far in the Monterey area have well-defined breeding seasons, and the majority spawn in the spring and summer months. In these cases, the free-swimming larval stages, unlike those of *Metandrocarpa taylori,* are plankton feeders which get into open waters where organic detritus is scarcer than near the shore, necessitating a reproductive cycle that is geared to the phytoplankton bloom.

I would like to thank Dr. Donald P. Abbott for the valuable criticism and advice he has given throughout the course of this work. I am indebted to Mrs. Emily Reid for preparation of many of the graphs.

SUMMARY

1. *Metandrocarpa taylori* Huntsman (1912) is an ovoviviparous colonial ascidian with separate zooids connected by a common basal tunic, which reproduces asexually by pallial budding.

2. Specimens in collections from field populations show maximum periods of sexual reproduction during the summer months. Although approximately 80% of the 1961 summer samples were reproductively active during this period, sexual reproduction continued during the remainder of the year with no less than 30% of the population active at any time. Eggs and testes develop simultaneously within the individual zooids.

3. Asexual reproduction was quantitatively studied in colonies cultured in the intertidal zone. Budding rates are lowest during the summer and continue at a high level during the remainder of the year. These rates are not dependent upon the size or age of the colony, but do derive from variations in rates at which successive bud generations follow one another and from the number of bud offspring produced by each zooid.

4. Although the two modes of reproduction are not mutually exclusive, it is clear that one form predominates in activity during any single season.

5. There is a general direct relationship between sexual reproductive activity and ocean temperature. There is little correlation of reproductive activity with food supply, since either phytoplankton or suspended organic detritus are abundant in Monterey Bay all year.

LITERATURE CITED

Abbott, D. P., 1953. Asexual reproduction in the colonial ascidian *Metandrocarpa taylori* Huntsman. *Univ. Calif. Publ. Zool.*, **61**: 1–78.

Abbott, D. P., 1955. Larval structure and activity in the ascidian *Metandrocarpa taylori*. *J. Morphol.*, **97**: 569–594.

Berrill, N. J., 1951. Regeneration and budding in tunicates. *Biol. Rev.*, **26**: 456–475.

Bolin, R. L., and D. P. Abbott, 1963. Studies on the marine climate and phytoplankton of the central coastal area of California, 1954–1960. *Calif. Coop. Oceanic Fish. Invest. Rept.*, **9**: 23–45.

Boolootian, R. A., A. Farmanfarmaian and A. C. Giese, 1962. On the reproductive cycle and breeding habits of two western species of *Haliotis*. *Biol. Bull.*, **122**: 183–193.

Boolootian, R. A., A. C. Giese, A. Farmanfarmaian and J. Tucker, 1959. Reproductive cycles of five west coast crabs. *Physiol. Zool.*, **32**: 213–220.

Diehl, M., 1957. Die Ökologie der Ascidie *Styela coriacea* in der Kieler Bucht. *Kieler Meeresforschungen*, **13**: 59–68.

Giese, A. C., 1959. Comparative physiology: annual reproductive cycles of marine invertebrates. *Annu. Rev. Physiol.*, **21**: 547–576.

Glynn, P. W., 1965. Community composition, structure and interrelationships in the marine intertidal *Endocladia muricata—Balanus glandula* association in Monterey Bay, California. *Beaufortia*, **12**(148): 1–198.

Goodbody, I., 1961. Continuous breeding in three species of tropical ascidians. *Proc. Zool. Soc. London*, **136**: 403–411.

Haven, N. D., 1965. Temporal patterns of sexual and asexual reproduction in the colonial ascidian *Metandrocarpa taylori* Huntsman. *Ph.D. thesis, Stanford University*, 163 pages.

Huntsman, A. G., 1912. Holosomatous ascidians from the coast of western Canada. *Contrib. Can. Biol.*, **1906–1910**: 103–185.

Levine, E. P., 1962. Studies on the structure, reproduction, development and accumulation of metals in the colonial ascidian *Eudistoma ritteri* Van Name, 1945. *J. Morphol.*, **111**: 105–138.

Lützen, J., 1960. The reproductive cycle and larval anatomy of the ascidian *Styela rustica* (L.). *Vidensk. Medd. Naturhist. Foren. Kjobenhavn*, **123**: 227–235.

MacGinitie, G. E., 1937. The use of mucus by marine plankton feeders. *Science*, **86**: 398–399.

Millar, R. H., 1954a. The breeding and development of the ascidian *Pelonaia corrugata* Forbes and Goodsir. *J. Mar. Biol. Ass. U. K.*, **33**: 681–687.

Millar, R. H., 1954b. The annual growth and reproductive cycle of the ascidian *Dendrodoa grossularia* (van Beneden). *J. Mar. Biol. Ass. U. K.*, **33**: 33–48.

Newberry, A. T., 1965. The structure of the circulatory apparatus of the test and its role in budding in the polystyelid ascidian *Metandrocarpa taylori* Huntsman. *Acad. Roy. Belg. Cl. Sci. Mem. Collect. Quarto.*, **16**(5): 1–57.

Orton, J. H., 1920. Sea-temperature, breeding and distribution in marine animals. *J. Mar. Biol. Ass. U. K.*, **12**: 339–366.

Ritter, W. E., and R. A. Forsyth, 1917. Ascidians of the littoral zone of southern California. *Univ. Calif. Publ. Zool.*, **16**: 439–512.

Sabbadin, A., 1957. Il ciclo biologico di *Ciona intestinalis* (L.), *Molgula manhattensis* (DeKay) e *Styela plicata* (Lesueur) nella laguna Veneta. *Arch. Oceanogr. Limnol., Venezia*, **11**: 1.

Thorson, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates, with special reference to the planktonic larvae in the sound (Öresund). *Medd. Komm. Danmarks Fiskeri Havundersogelser Ser. Plankton*, **4**(1): 1–523.

Trason, W. B., 1963. The life cycle and affinities of the colonial ascidian *Pycnoclavella stanleyi*. *Univ. Calif. Publ. Zool.*, **65**: 283–326.

Reference: *Biol. Bull.*, **140**: 416–426. (June, 1971)

AN ATTEMPT AT A SUBJECTIVE AND OBJECTIVE CLASSIFICATION OF THE ACIDOPHILIC GRANULOCYTES OF SOME MARINE FISHES[1]

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The study of the acidophils in the blood smears of fishes has been very limited. Drzwena (1911) in the most extensive study of the morphology and tinctorial reactions of the acidophils of the blood smears of fishes, fixed by heat and stained with acid or basic dyes, concluded that the acidophils or eosinophils are those cells in which the granules stain only with the acid dye. She distinguished a tinctorial difference from light orange (indicative of faint eosinophilia) to deep wine red (indicative of strong eosinophilia). The cells could be of any size or shape, the nucleus of any shape, the granules of any size or shape, and there could or could not be a basophilic matrix. She studied the eosinophils of the Chondrichthyes and Osteichthyes and found great variation in the sizes and shapes of the granules and their tinctorial reactions even in closely related species. In this report I am presenting evidence for the classification of such cells not only by subjective description, but by their transmission of monochromatic light.

The basic assumption upon which this report is based, is that if monochromatic light is passed through a film or filter with the same color as the wave length of the light source, the light passed through will have the same value except for some reflection or absorption depending upon the material of the filter (Weiskopf, 1968).

From a study of the objective types of acidophils it was hoped that the objective measurements of the degrees of transmission of monochromatic red light could be made directly on the stained cells. However, it was found that in order to get sufficient magnification to do this, the heat transmitted at the same time was so great that the dyes faded rapidly. Consequently, it was decided to make an objective classification of these subjectively classified acidophils by measuring the amount of monochromatic red light transmitted through photomicrographs of acidophils which had been taken at oil immersion magnification (1250 ×, reduced for photography by $\frac{1}{3}$ by the Leitz adapter) on color film, which in turn was magnified by 41 ×, so the total magnification of the cytoplasm of the acidophils at which the transmission was measured was 16,400 ×.

The objective measurements of the acidophilic material of the acidophils studied by this method when analyzed statistically showed a variety of differences in transmission by acidophils with similar subjective characteristics. It it suggested that these differences, which are based upon transmission by human eosinophils as a control, may be used as an auxiliary tool in taxonomic classification and in experimental investigation. It is hoped that some method based upon this may be devised which is similar to the physical methods used by Stein, Lipkin, and Shapiro (1969) to facilitate optical measurements in biology.

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METHODS

The methods of study are simple, but the need for more accurate preparations of the materials for the present observations were not realized until after the blood smears had been stained, and photographs made at different times, on different films. The absolute identity of preparation could not be done, because of variations in the amount of plasma clinging to the cells, the difference in density and probably in chemical content of the plasma, the degree of flattening of the cells in making the smears under varied environmental conditions by different people, and the possible difference in the chemical characteristics of the Wright's stain over the period of several years when the smears were collected and stained. An attempt was made to control such things as could be controlled. The air-dried smears were exposed to 10 drops of Wright's stain for one minute, and a similar amount of distilled water buffered to pH 6.8 was mixed with the stain and allowed to stand for two minutes; the water was rinsed off, the stained smear was dried rapidly in air and stored. This is a standard method for the preparation for study of the blood cells of man, but as pointed out above there were difficulties in staining adsorption because of the plasma. Apparently this did not affect the leucocytes, but erythrocytes often tended to be more blue than red.

The photomicrographs were made on the same Leitz Aristophot under oil immersion at a magnification of 1250 ×, reduced by the $\frac{1}{3}$ adapter. The source of light for photography was 1.2 foot candles with daylight (80C) filter, and the exposure time was $\frac{1}{5}$ second on Ektachrome F (speed 16), and $\frac{1}{10}$ of sec with daylight (85) filter on Ektachrome X (speed 64).

Since the observations on the blood cells of the fishes were part of the larger project on the morphology of the blood cells of the vertebrates, an attempt was made to photograph sample cells from each of the fishes. After preliminary study in which counts and descriptions of the acidophilic granulocytes were made from identifications given in the literature by Drzwena (1913) and other investigators and reviewed by Jordan (1938), Kindred (1961), and more recently by Saunders (1966), the photomicrographs were made for this projected atlas. It is from these photomicrographs that the following contrasts between subjective characteristics and objective measurements of transmission of light by the acidophilic materials have been made.

First, the subjective classification has been made on the basis of the presence of acidophilic material alone in the cytoplasm (type A); acidophilic materials and clear spaces (type B); acidophilic materials and basophilic matrix (type C); and acidophilic materials, clear spaces and basophilic matrix (type D). Next, the objective characteristics, the transmission of monochromatic red light (Filter 29W)/1.0 mm^2) by the largest possible area of the cytoplasm was measured as delineated by a masked strip placed over the photomicrograph of the cell as it was viewed on the microscopic stage at a magnification of 41 ×. Since the photomicrograph had been made at a magnification of 400 ×, the total magnification of the viewed cell was 16,400 ×. Specially prepared diagrams of the areas viewed permitted rapid recording of the photometric measurement of the localized transmission by placing the scanner over the ocular. Since the amount of light passing through the photomicrograph depended upon the area delineated and on the relative amounts of acidophilic material, clear areas, and basophilic matrix, the percentage of each of these areas was measured on projected photomicrographs at a magnification of 5600 ×. The difference between the amount of light transmitted by the photographic film and layer of plasma on the same photomicrograph in an area equal to the extent to that of the cytoplasm measured, and that by the photographic film, plasma and cytoplasm of the cell, gave the actual measurement of transmission by the area of the cytoplasm alone. The role of the different tinctorial reactive areas in the total measurement was then calculated from the ratio of transmission by acidophilic material, clear areas and basophilic matrix to each other. These fundamental data were obtained by measurement of the transmission of the red light from the light source (0.83 foot candles/1.0 mm^2), that transmitted by clear film for the clear area (1.2 ft-c/1.0 mm^2), and for the basophilic matrix (0.22 ft-c/1.0 mm^2). From these data the amount of red light transmitted for each tinctorial area of the cell was calculated and entered in a master table from which only the transmission by the acidophilic area is entered in Figure 1.

Finally, for statistical analysis, the mean transmission of red monochromatic light by the acidophilic elements of eight photomicrographs of human eosinophils, stained with Wright's stain by the same method given above (0.12 ft-c/1.0 mm^2) was used as a control. The X2 deviations from this control by the measurements of transmission by the fish acidophils were used to classify the acidophils objectively (Fig. 1) (Fisher, 1934; Croxton, 1953).

Since the photomicrographs had been taken on either Ektachrome F (speed 16) or Ektachrome X (speed 64), allowing for difference in speed as stated above, the transmission by the photomicrographs of the cells on the two types of film were tested for bias. These calculations indicated that there was no significant bias in the tinctorial reactions of the acidophilic contents, and such deviations as occurred seemed to be dependent on the adsorption of the dye by the acidophilic granules of the cells which had been photographed rather than on the reactions of the film used.

These data and the data on the details of the measurements of the sizes of the cells, areas and transmission by all parts of the cells, calculations for correlations, size, weighting of the samples, and other such basic data which seemed too cumbersome and nonessential for publication in such a pilot study are filed if wanted for further reference. The chief results of pertinence are presented in Figure 1 which depicts graphically the transmissions in their relations to the species of fish in which they occur, and the significant deviations of the transmissions from the control by the different types of cells. The arrangement of the species by families followed chiefly the classification of marine fishes given by Perlmutter (1961), supplemented by reference to classifications given by Smith (1898), Collins (1959), Herald (1961), and Hvass (1965).

RESULTS AND DISCUSSION

Before going into a detailed examination of the significance of the results I wish to point out that an attempt has been made in Figure 1, to arrange the data so that the chief results of the investigation may be seen as a whole. Thus it is seen that there are cells from only one species in type A, that most of the cells in type B are in the Chondrichthyes, and most of the cells of type C are in the Osteichthyes. Except for one cell in the Marsipobranchii, all of the cells of type D are in the Osteichthyes. Not only are these distributions quite apparent by casual perusal of the chart, but the trends of the transmissions with reference to the control may be easily seen. With these facts in mind the data are now examined to see whether the method has possibilities for use as a taxonomic or experimental tool. In the following analysis of the data, the references to categories are kept as brief as possible. References to families are not entered in the chart, but are in parenthesis following the species name.

Marsipobranchii

There are no acidophile of types A or C in this class.

Type B. There is one measurement in *Petromyzon marinus* (Petromyzontidae), not significantly different from the control; and one from *Myxine glutinosus* (Myxinidae) with a significantly lower transmission.

Type D. One measurement from *Petromyzon*, significantly higher than the control. From these scant data there is a suggestion of significant differences in the acidophilic material of the two species probably caused by differences in density of the acidophilic material. There is however, no great diversity between the transmissions such as will be seen in these types of acidophils in the species of the other classes.

Chondrichthyes

Type A. Three measurements from *T. torpedo* (Torpedinidae), none of which is significantly different from the control.

Type B. Three measurements from *T. torpedo*, all significantly lower than the control. Two measurements from *Squalus acanthias* (Squalidae) both significantly lower than the control. Two measurements from *Raja erinacea* (Raidae), both significantly lower; four measurements from *R. laevis*, one not different, and three significantly lower; and two measurements from *R. radiata*, one not different, and one significantly lower than the control. Thus of the 13 measurements made only two (15%) are not different from the control; and eleven (85%) are significantly lower. There were none higher than the control. Hence, the data on these cells are regarded as giving evidence of a denser content and greater adsorption of the acidophilic dye than occurs in the acidophilic granules of the human eosinophils used as a control. The transmissions do not permit any evidence for familial or specific differences within the class, but they do indicate that the transmissions are significantly low and are fairly constant through-out the class.

Type C. Seven measurements from three species, from three families were made of this type. In five measurements from *Scyliorhinus stellatus* (Scyliorhinidae), all are signficantly higher than the control. In *Mustelus canis* (Triakidae) with one measurement, the transmission is significantly higher than the control. Also in *R. ocellata*, the one measurement is significantly higher. It is to be noted that in type C, there are no measurements significantly lower than the control. This is the reverse of the trend of the transmissions by type B and the difference is regarded as of value for use in discriminating between the species. One would expect the transmission to be lower in type C than B, because of the presence of basophilic matrix, but none of these measurements is lower than the control, all are higher. It would appear that this objective method shows up differences clearly in these Chondrichthyes.

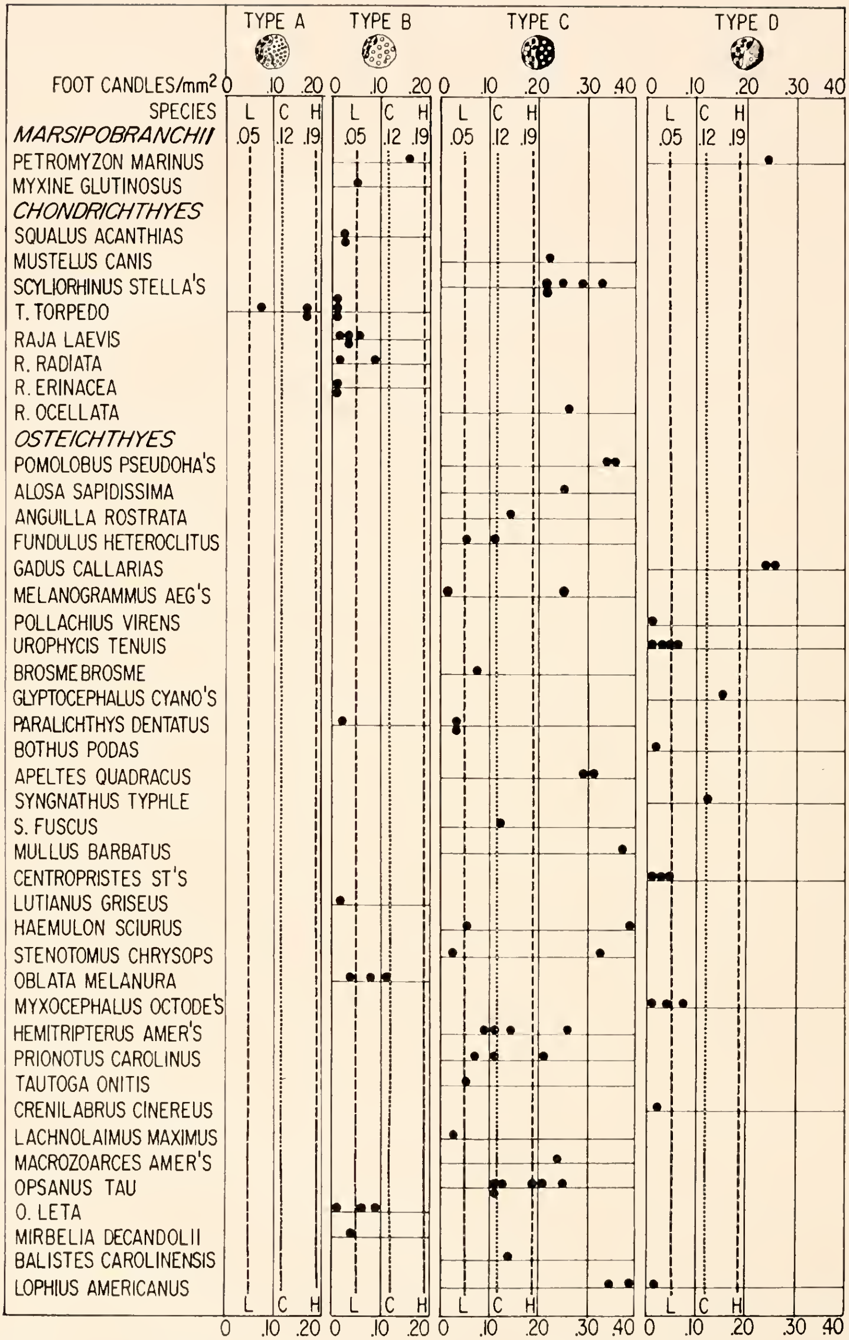

FIGURE 1. Acidophils of marine fishes. A chart showing the distributions of the transmission of monochromatic (red) light by the acidophilic areas of photomicrographs in foot

In looking over the transmissions by all type of cells in the Chondrichthyes, it can be seen that the transmission by cells of type A are not significantly different from the control, those of type B in which the largest number of species were studied and measurements made, have a greater number of transmissions significantly lower than the control; and in type C this trend is reversed.

Type D. There are no cells of type D present.

Osteichthyes

Type A. There are no cells of this type in the Osteichthyes.

Type B. There are only five species with acidophils of this type. When the distributions of the several measurements are examined, it is seen that in *Paralichthys dentatus* (Paralichthydae), the one measurement is significantly less than the control; of the three measurements in *Oblata melanura* (Sparidae) two are not different and one is lower than the control; in *Mirbelia decandolii* (Gobiesocidae), the one measurement is significantly lower; as is also the one measurement in *Lutianus griseus* (Lutianidae); and in three measurements from *Opsanus leta* (Batrachioididae), two are not significantly different from the control and one is lower. Of the nine measurements, four (45%) are not different from the control, and five (55%) are significantly lower, and none is higher. The cells of type B are not characteristic of the Osteichthyes and occur in the smallest number of species. Theoretically, because of the presence in most of the cells of a high percentage of clear area, the transmissions should be higher than lower.

Type C. This type of acidophil is characteristic of the Osteichthyes. It has been observed in twenty species from seventeen families, and is usually the only type of acidophil present in these species. Following down the list of measurements and their deviations from the control in Figure 1, it is seen that in *Pomolobus pseudoharengus* (Clupeidae) both measurements are significantly higher than the control; and in *Alosa sapidissima*, in the same family, the single measurement is also higher. In *Anguilla rostrata* (Anguillidae) the single measurement is not different from the control; in *Fundulus heteroclitus* (Poecillidae) both measurements are not different from the control; in *Melanogrammus aeglefinis* (Gadidae), one is higher and one is lower; and in *B. brosme* (Gadidae) the single measurement is not different; in *Paralichthys dentatus* (Paralichthydae) both measurements are significantly lower; in *Apeltes quadracus* (Gasterosteidae) both are significantly higher; in *Syngnathus fuscus* (Syngnathidae) the one measurement is not different from the control; in *Mullus barbatus* (Mullidae) the one measurement is significantly higher than the control; in *Haemulon sciurus* (Haemulidae), of the

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candles/1.0 mm^2. At left, names of classes and species arranged in taxonomic order; four columns for transmissions by subjective types A, B, C and D arranged by foot candles/1.0 mm^2 from 0 to the highest transmission for each type; above each column is a diagram showing the distributions of the tinctorially visible areas in each type, acidophilic granules or vesicles (outlined white), clear areas (white not outlined) and basophilic areas, black. The dotted line at 0.12 ft-c (C) is location of control transmission for each type; dash lines at 0.05 (L) and 0.19 (H) ft-c are the lower and upper limits, respectively, of measurements not significantly different from the control as calculated by the X2 method for agreement; to the left of L, the measurements are significantly lower, and to the right of H, they are significantly higher than the control; solid circles, position of transmission for each measurement; horizontal lines, projection of the position of the species in relation to the type of cell.

two measurements, one is not different, and the other is significantly higher than the control; in *Stenotomus chrysops* (Sparidae), one is lower and one is higher; of the four measurements in *Hemitripterus americanus* (Hemitripteridae), three are not different and one is significantly higher than the control; of the three measurements in *Prionotus carolinus* (Triglidae), two are not different, and one is significantly higher. Of the two species of Labridae, the one measurement in *Tautoga onitis*, is not significantly different; and that from *Lachnolaimus maximus* is significantly lower than the control. The one measurement from *Macrozoarces americanus* (Zooarchidae) is significantly higher than the control. Of the six measurements from *Opsanus tau* (Batrachioididae) four are not significantly different from the control, and two are significantly higher. The single measurement from *Balistes carolinensis* (Balistidae) is not significantly different from the control. Of the two measurements from *Lophius americanus* (Lophidae) both are significantly higher than the control.

Reviewing and summarizing these distributions it is seen that of the 38 measurements made from twenty species having acidophils of type C, seventeen (45%) are not significantly different from the control; five (13%) are significantly lower, and sixteen (43%) are significantly higher. Examination of the deviations in the X2 columns, shows that there is not much deviation by the cells with low transmissions, while those with the higher transmissions have much greater deviations. When we localize these figures for species by using the transmission as a measure of classification, it is seen that there are six species having only acidophils not significantly different from the control; *Anguilla*, *Fundulus*, *Brosme*, *S. fuscus*, *Tautoga*, and *Balistes*. There are two species with only acidophils with transmissions significantly lower than the control, *Paralichthys* and *Lachnolaimus;* and six species with only acidophils with significantly higher transmission, *Pomolobus*, *Alosa*, *Apeltes*, *Mullus*, *Macrozoarces* and *Lophius*. Of the species in which variations of transmission occur, there are three species in which there are more cells with transmission not significantly different and with significantly higher transmission, (ratio in parenthesis); *Hemitripterus* (3:1), *Prionotus* (2:1), *Opsanus tau* (5:1); and finally, there are two species, *Melanogrammus* and *Stenotomus*, in which no non-significantly different cells occur and the ratio of significantly lower to significantly higher is equal.

Thus it would seem that in these cells of type C, there is a much greater tendency for the transmission not to be different from the control, a slight tendency for the cells to have a low transmission, and great tendency to deviate to a higher transmission. The deviation to higher and lower transmissions are suggested to be indications for significant modification of the acidophilic substance in these species, a modification which could not be detected without the use of the present method.

When the distributions of the cells of type B and their transmissions in the Osteichthyes are contrasted with type B of the Chondrichthyes, the most striking difference between them is the absence of cells with significantly higher transmission in the Chondrichthyes, and the tendency for most of the cells to have significantly lower transmissions than the control.

Type D. It should be recalled that the cytoplasm of the acidophils of this type contains a mixture of clear areas, and basophilic matrix, as a background for the acidophilic granules or vesicles. The areas occupied by these materials were taken into account in calculating the transmissions. There are ten species from eight families which have cells of this type. There is one family (Gadidae), in which three species were studied. Of the two measurements in *Gadus callarias* both are significantly higher than the control; the single measurement from *Pollachius virens* is significantly lower, while of the four measurements from *Urophycis tenuis*, two are not significantly different from the control; and two are significantly lower. In *Glyptocephalus cyanoglossus* (Pleuronectidae), the single measurement is not significantly different; in *Bothus podas* (Bothidae) the transmission is significantly lower; and in *S. typhle* (Syngnathidae) it is not significantly different; in *Centropristes striatus* (Serranidae), of the three measurements, all are significantly lower than the control; and in *Myxocephalus octodecimspinosus* (Cottidae) of the three measurements, two are not significantly different and one is significantly lower than the control; in *Crenilabrus cinereus* (Labridae) the single measurement is significantly lower than the control; as is the single measurement in *Lophius americanus*.

The distribution of these measurements in type D shows that the greatest number of the measurements are significantly lower than the control and the least number are significantly higher. These deviations are different from the trend toward higher measurements in type C.

It would seem that by this method the differences within the types can be used as a basis for objective classification of the species in which they occur. There are a few species in which cells of more than one type are present. In *Petromyzon* there are cells from types B and D; in *Torpedo* from A and B; in *Paralichthyes*, from B and C; and in *Lophius*, from types C and D.

Review of the distributions by class, type and transmission

Total number of species, 43. Distributions of the types by species in the several classes is as follows: (percentage of total number of species in parenthesis):

Marsipobranchii. Two species (4.7%); type A, none' type B one (2.3%), not different (2.3%); type C none; both type B and D, one (2.3%), not different from control and higher.

Chondrichthyes. Eight species (18.5%); type A and B in same species, one (2.3%); transmission lower and not different from control (2.3%); type B, four species (9.4%), two lower (4.7%); two not different and lower (4.7%); type C. three species (7.0%), all higher (4.7%), than the control; type D, none.

Osteichthyes. Thirty-three species (77%); type A, none; type B (only) four (9.4%), two not different and low (4.7%), two low (4.7%); type B and C in same species, one (2.3%), low (2.3%); type C alone, eighteen (42%); six not different from control (14.1%), three lower (6.2%), five higher (11.5%), three not different and higher (6.9%); type C and D in the same species one (2.3%), transmission lower and higher (2.3%); type D alone, nine species (21%); in two (4.7%), transmission not different from control, four (9.4%) lower than control, one (2.3%) higher, two (4.7%) transmission not different and lower in same species.

The distribution by type throughout the whole forty-three species is: type A only, none; type B only, ten (23%); type C only, twenty (46%); type D only, nine (21%); types A and B in one species, one (2.3%); B and C in one species, one (2.3%); B and D in one species, one (2.3%); and C and D in one species, one (2.3%).

Finally out of all the species in the different classes, the distribution of the types is: in the Marsipobranchii, two species, of which one (50%) has type B cells only; and the other has type B and D cells (50%); in the Chondrichthyes (eight species); types A and B in the same species, one (12.5%); type B only, four (50%); type C only, three (37.5%); and type D only, none. In the Osteichthyes, thirty-three species: type A, none; type B, four (12.1%); type C, eighteen (54.5%); type D, nine (27.2%); types B and C in the same species, one (3.2%); types C and D in the same species, one (3.2%). In the two classes in which there are more than three measurements, the incidence of types B and D are highest in the Chondrichthyes; and types C and D, in the Osteichthyes. The latter two types of acidophils differ from the others by the presence of basophilic matrix, which material may be taken as the characteristic substance of the acidophils of the Osteichthyes.

This investigation was undertaken as a pilot project to supplement the descriptions of the blood cells of fishes which I had made as part of a wider survey of the morphology of the blood cells of the Vertebrates (Kindred 1961). The blood cells had been photographed and described by the usual subjective methods, when in reviewing the material, I thought that an objective numerical value entered beside the subjective description, would be of great help in making comparisons between the characteristics of the blood cells of the different fishes. In this paper I have presented the result of such an attempt. I have obtained such objective data, but the question arises whether they can be used effectively in their present form, knowing that the preparation of the materials has been handicapped by the presence of some modifying factors; such as the method of preparation of the smears, thickness of the smears, amount of the plasma clinging to the smears, the chemical characteristics of the Wright's stain used, the degree of adsorption of the dyes and the uniformity of the photomicrographs. However, since it is known that these factors are not particularly taken into consideration at present in making morphological descriptions of the blood cells such as occur in the literature of the hematology of the leukocytes, it was decided to present my results as a preliminary pilot study which could be followed by a wider and more accurate evaluation of the problems involved in achieving the results. Thus there is a place in the methods of study of the blood cells under the present conditions which I hope I have partially filled by showing that objective results can be obtained, when the materials are prepared under the present methods, taking such precautions to influence uniformity as can be taken. The present objective results emphasize definite trends in the transmission of red light by certain tinctorial areas of the acidophils which may be utilized as objective differences between the acidophils of the different species of fish and which may be of taxonomic value in assessing differences between species or in changes in the acidophils brought about by experimentation.

The fishes furnished by the U. S. Bureau of Commercial Fisheries, Woods Hole, Massachusetts, were classified by Mr. Kenneth Cumming; fishes of Naples, Dr. P. Tardent of the Zoological Laboratory; and the few species from Florida, by Dr. Leonard J. Greenfield of the Marine Laboratory of the University of Miami.

SUMMARY

Highly magnified colored photomicrographs of the acidophilic granulocytes stained with Wright's stain of forty-three species of marine fishes were studied in an attempt to relate subjective descriptions of the cells to objective measurements of the transmission of monochromatic red light by the acidophilic materials (rods, granules, or vesicles). Four types of acidophils are classified by the distribution of the acidophilic materials, clear spaces and basophilic matrix of the cytoplasm. Objectively these types are classified statistically by the X2 method for deviations from a control transmission obtained in this case from the mean transmission by acidophilic materials in photomicrographs of human eosinophils prepared and photographed under the same conditions as was done in the fishes.

The data are presented in a chart (Fig. 1) showing the class, and species from which the cells came; type of cell (A, B, C, or D); transmission of the red light in foot candles/1.0 mm^2 by the acidophilic materials; range of significant deviations from the control as calculated by the X2 method.

Briefly, cells with only acidophilic granules, are found in only one species, *T. torpedo,* of the Chondrichthyes and the transmission is not significantly different from the control. Cells of type B, characterized by acidophilic materials (granules, rods or vesicles) and clear areas, predominate in the Chondrichthyes, and most of them have significantly low transmission; a few species of Osteichthyes, have such cells, and the transmissions are sufficiently variable to justify the use of the objective data as a tool in classification. Cells of type C, with acidophilic materials (granules and vesicles) and basophilic matrix, predominate in the Osteichthyes. There are a few species of Chondrichthyes which have them and in most of these cells the transmission is significantly higher than the control. In the Osteichthyes, these cells are characteristic and in practically all species, the transmission is either higher than the control, or not significantly different. Very few species have cells with significantly low transmission. Again it may be pointed out that the objective differences in the cells of type C permit a classification which could be of taxonomic value. Finally, the cells of type D, with acidophilic vesicles, clear spaces and basophilic matrix are not represented in the Chondrichthyes. One sample was found in the Marsipobranchii, but all the rest are in species of Osteichthyes. In only one species is the transmission significantly higher than the control, the transmissions by the cells of the other species are either not significantly different from the control, or are lower.

It is concluded from this pilot study that the objective results obtained from measurements of transmission of monochromatic red cell light by the acidophilic components in photomicrographs of the acidophils of fishes stained with Wright's stain, to warrant its use with modification as a tool in the objective study of blood cells in smears.

LITERATURE CITED

COLLINS, H. H., 1959. *Complete Guide to American Wildlife.* Harper and Brothers, Philadelphia, 683 pp.

CROXTON, F. E., 1953. *Elementary Statistics with Applications in Medicine.* Prentice Hall, New York, 376 pp.

DRZWENA, A., 1911. Les leucocytes granuleux des Poissons. *Arch. Anat. Microscop.,* **193**: 319–376.

Fisher, R. A., 1934. *Statistical Methods for Research Workers.* [5th Edition] Oliver and Boyd, London, 319 pp.

Herald, E. S., 1961. *Living Fishes of the World.* Doubleday and Co., New York, 156 pp.

Hvass, H., 1965. *Living Fishes of the World.* E. P. Dutton Co., New York, 156 pp.

Jordan, H. E., 1938. Comparative hematology. *Section XII in Handbook of Hematology.* Hoeber, New York.

Kindred, J. E., 1961. Vertebrate blood. Pages 161–164 in *Encyclopedia of Biological Sciences.* Reinhold Publishing Co., New York.

Perlmutter, A., 1961. *Guide to Marine Fishes.* New York University Press, New York, 431 pp.

Saunders, D. C., 1966. Differential blood counts of 121 species of marine fishes from Puerto Rico. *Trans. Amer. Microscop. Soc.*, **85**: 127–449.

Stein, P. G., L. E. Lipkin and H. H. Shapiro, 1969. Spectre II, General-purpose microscope unit input for a computer. *Science*, **166**: 328–333.

Weiskopf, V. F., 1968. How light interacts with matter. *Scient. Amer.*, **219**: 60–71.

Reference: *Biol. Bull.*, **140**: 427–439. (June, 1971)

HABITUATION IN *LIMULUS* ABDOMINAL GANGLIA [1]

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The phenomenon of habituation, or response decrement with repetitive stimulation, is generally considered to be a form of learning and distinguishable from response fatigue and receptor adaptation (Bullock and Quarton, 1966). It may be argued that it is equally important for an animal to be capable of "learning not to respond" (Thorpe, 1966, page 60) as it is for the animal to acquire new responses or potentiate existing ones. Habituation may be a form of learning common to all organisms—its demonstration in *Phycomyces* (Ortega and Gamow, 1970), protozoa (Applewhite, Gardner and Lapan, 1969; Wood, 1970), coelenterates (Rushforth, 1967), planarians (Westerman, 1963), several arthropods (for examples and reviews, see McConnell, 1966; Thorpe, 1966; Thorpe and Davenport, 1965; Treherne and Beament, 1965) as well as in the vertebrates (Sharpless and Jasper, 1965; Thompson and Spencer, 1966) attests to a phylogenetic ubiquity.

In *Limulus polyphemus*, previous research has suggested that this "living fossil" also possesses the capacity for habituation of visual reflexes (Corning and Von Burg, 1968). Stimulation of a lateral eye elicits leg movements that are mainly contralateral. With repeated stimulation, the frequency of reflex activation was found to diminish. Evidence for a central locus of the response decrement was obtained when tests of the unstimulated eye demonstrated an initially lower probability of response elicitation in the opposite set of legs. Attempts to demonstrate other types of learning in *Limulus* have been negative or not overly convincing (Makous, 1969; Smith and Baker, 1960; Wasserman and Patten, 1969).

In the present investigations, we have attempted to obtain electrophysiological evidence of habituation in *Limulus* by studying the effects of repetitive tactile stimulation on the activity of abdominal ganglia efferents. The abdominal ganglia of *Limulus* provide an experimentally amenable system for studying habituation and its underlying mechanisms (Von Burg and Corning, 1969; 1970). The entire ventral cord region can be exposed in 15–20 minutes, and the preparation is viable for at least 6 hours although in some cases it has been electrophysiologically active for 24–48 hours. The first four abdominal ganglia are large and anatomically differentiated, permitting lesions and electrode placements to be made at readily identifiable sites.

Each ganglion has two pairs of nerves: dorsal and ventral. The ventral nerves carry efferents that control gill movement (Hyde, 1893), and afferents from the surfaces of the gill books and carapace (Patten and Redenbaugh, 1900). The dorsal nerves contain cardioinhibitors and cardioaccelerators as well as sensory fibers from the lateral spines (Corning, Von Burg and Lahue, 1970; Von Burg and Corning, 1970). Tactile stimulation of the gill books will produce a marked increase in dorsal nerve output and a transient inhibition in heart rate. In the intact system, tactile stimulation will affect the activity of dorsal nerves in the other abdominal ganglia as well as the output of the ipsilateral and contralateral

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dorsal nerves in the same segment. However, the sensory information carried by afferents in each ventral nerve is derived solely from that segment and on the side that is stimulated. Thus, sensory information is segmental in origin whereas the consequences of this activation appear to be diffused throughout the central ganglia.

The direct examination of habituation in the central nervous system permits a more precise definition of afferent and efferent bioelectrical activity during stimulation. Presumably, if habituation is a central phenomenon, repetitive stimulation should produce response decrements in the ganglion outputs and have little effect on the activity of the sensory side. In addition, by studying the arthropod central nervous system with its segmented ganglionic masses, it is possible to examine the habituation process in the single ganglion, groups of ganglia, and in the intact system. These types of analyses can provide information concerning the relationship between neurological complexity and the acquisition and retention characteristics of the habituation process.

METHODS

Adult male and female specimens of *Limulus polyphemus* were procured from the Marine Biological Laboratory, Woods Hole, Massachusetts, April through December. The animals ranged from 6–8 inches in width as measured across the widest part of the cephalothorax. They were held in tanks of artificial salt water ("Instant Ocean") at a specific gravity of 1.022, pH of 7.8–8.3, and temperature of 15 to 18° C. The water was constantly filtered and for 20 min every hour there was heavy aeration.

For experimentation each subject was affixed, dorsal side down, to a board. This was most easily accomplished by driving nails through the peripheral edges of the carapace into the board. All walking legs were removed at the coxal-trochanter joint. This is necessary to reduce movement artifact during recording, and to prevent the legs from interfering with the electrodes or stimulating apparatus. The gill books were then split medially and the ventral surface of the opisthosoma opened along the midline from the last gill book forward past the operculum. The abdominal endochondrites were sectioned and, after lateral displacement of muscle and connective tissue, the ventral cord was completely exposed.

All recordings were made from the dorsal and ventral roots of the first four abdominal ganglia. Measures of ganglion output were made in dorsal nerve efferents. These are very responsive to tactile stimulation of the hair bristles along the ventral surface of the animal and the gill book surfaces. To obtain reliable recordings of the activity in these roots it was necessary to remove the membrane around the nerve. An incision was made with an eye scalpel and the membrane peeled away with fine dissecting forceps. A small portion of the root was nipped and the proximal end drawn into a suction electrode with an opening of approximately 10–15 μ. Tactile information enters the abdominal ganglia via the ventral roots. When recording the degree of afferent activity elicited by stimulation, the ventral nerve was dissected free to a point just before it entered the gill book. At this point the nerve was severed and the distal end of the bundle was drawn into a suction electrode.

Stimulation was provided by a light puff of air directed upon the ventral surface of the gill books through a glass pipette with a 1 mm tip diameter. The pipette tip was usually 5–7 mm from the gill book surface. Air pressure was provided by an air pump and the duration of the air flow was controlled by an Asco 2-way air valve activated by a square wave stimulator. The air puff was 0.5 sec in duration and was delivered once every 0.73 sec. Each animal received 3 blocks of stimulation with each block consisting of 360 puffs. The first block is designated as an "acquisition" phase. Three minutes after the termination of the first block, a second series of 360 puffs was delivered; this block is referred to as the "3-min retention test." Nine minutes after the completion of the second block the third block, or "9-min retention test," was initiated. At the completion of the last air puff for each block the stimulated point was given one of three "dishabituation" stimuli: a drop of water, a stronger puff of air, or a touch with a blunt dissecting probe. These stimuli provided tests for fatigue, *i.e.*, if response decrements represent true habituation, then stimuli of different intensity or quality should still be effective.

Four major preparations were studied. One group (14 animals) was habituated with a totally intact central nervous system. The stimulating and dorsal nerve recordings were accomplished at the same segment as well as different segments. However, no more than two ganglia intervened between the afferent input and the dorsal nerve that was recorded since the experiments were limited to the first four abdominal ganglia. A second group (15 animals) was habituated with the ventral cord region isolated from the rest of the central nervous system. As in the intact group, stimulation and recording were carried out at the same segment as well as over different segments. In a third group (14 animals), habituation was attempted in a ganglion that had been completely isolated from the central nervous system. In this group, the stimulation and recording were necessarily restricted to the same neuromere. To determine whether a response decrement in a ganglion output could be due to sensory adaptation or fatigue, recordings were made in sensory branches of the ventral nerve that innervated the gill book surface. In these preparations (4 animals), the mode and frequency of stimulus delivery were the same except that the recordings were made in the afferent fibers.

The bioelectrical activity was amplified with a Grass P511 ac amplifier with a HIP-511C high-impedance probe and stored on magnetic tape for later analysis. Unit activity was counted with a Ferch variable voltage gate and Hewlett-Packard high-speed counter. A Honeywell Model 906C Visicorder recorded both the raw data and the gate output simultaneously for a visual record of the spikes being counted during a stimulus interval (0.73 sec). Before each series of 360 stimulus presentations, the spontaneous discharge rate was sampled for ten 0.73 sec intervals. The average count was then subtracted from the counts recorded during each stimulus presentation. This procedure corrected for any shifts in the spontaneous firing rate during the experiment. The Mann-Whitney U-Test and Wilcoxon Matched-Pairs Signed-Rank Test (Siegel, 1956, pages 75 and 116) were used to assess the statistical significance of any response change. A reference to "statistical significance" indicates a probability error of 0.05 or less (two-tailed test).

RESULTS

Response decrements during acquisition

The initial responses to the first few air puffs were marked, but the number of spikes decayed rapidly during the first minute of stimulation. Most of the response decrement in all preparations took place during the first 60 puffs. This period is plotted in Figure 1 ("acquisition") for the three major groups. The

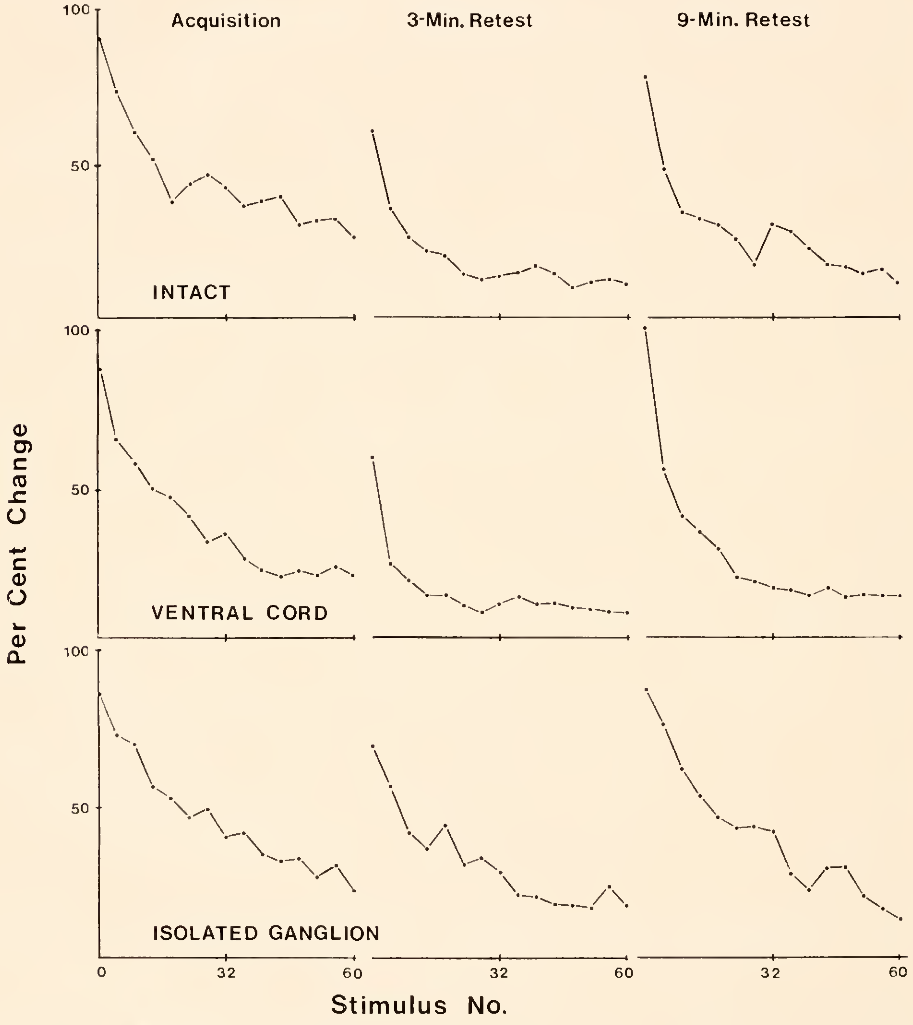

FIGURE 1. Median per cent changes in unit counts for the first 60 air puffs in all animals. The data are represented as per cent values; the initial (maximal) spike count recorded during the first air puff was used as the reference point (100%). All counts have been corrected with respect to any shifts in the baseline discharge rate recorded between series of stimulus presentations.

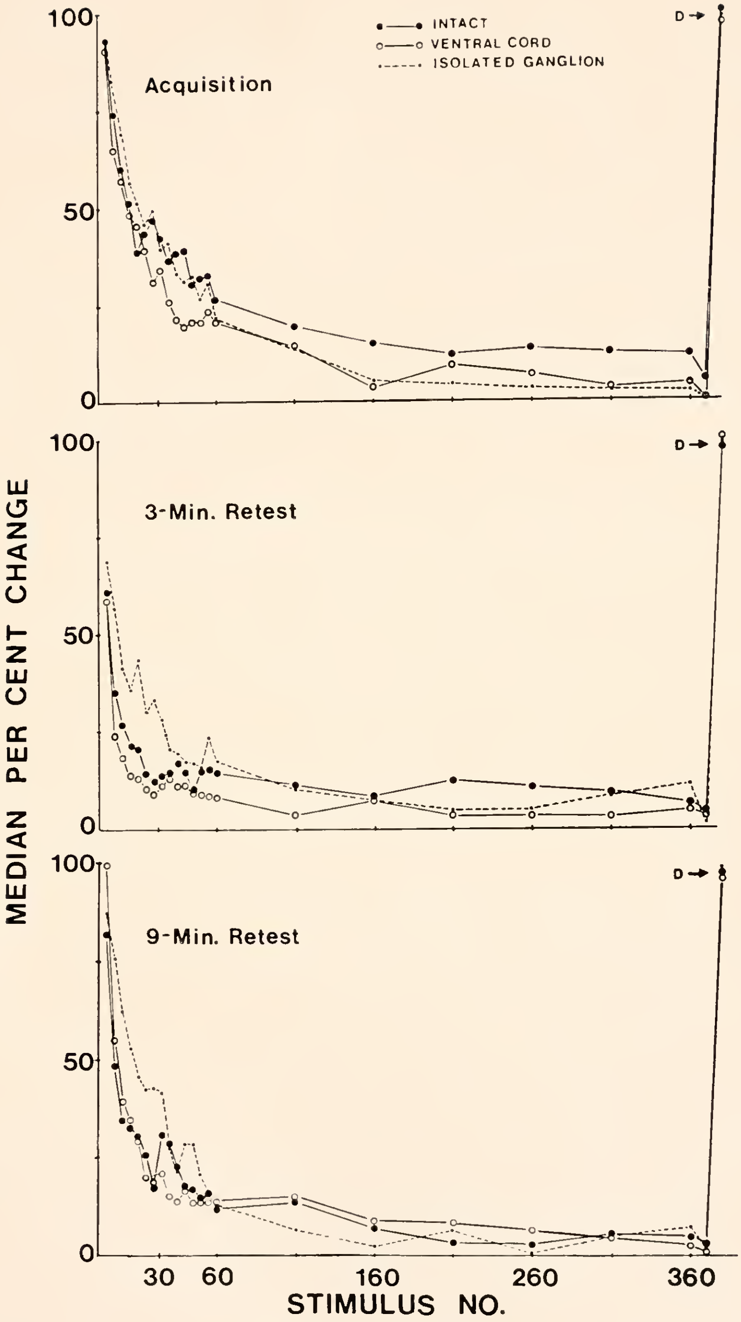

FIGURE 2. Comparisons of the Intact, Ventral Cord, and Isolated Ganglion groups during acquisition and retesting. "D" indicates the application of a different stimulus (usually a water drop) to the area that had been receiving the air puff.

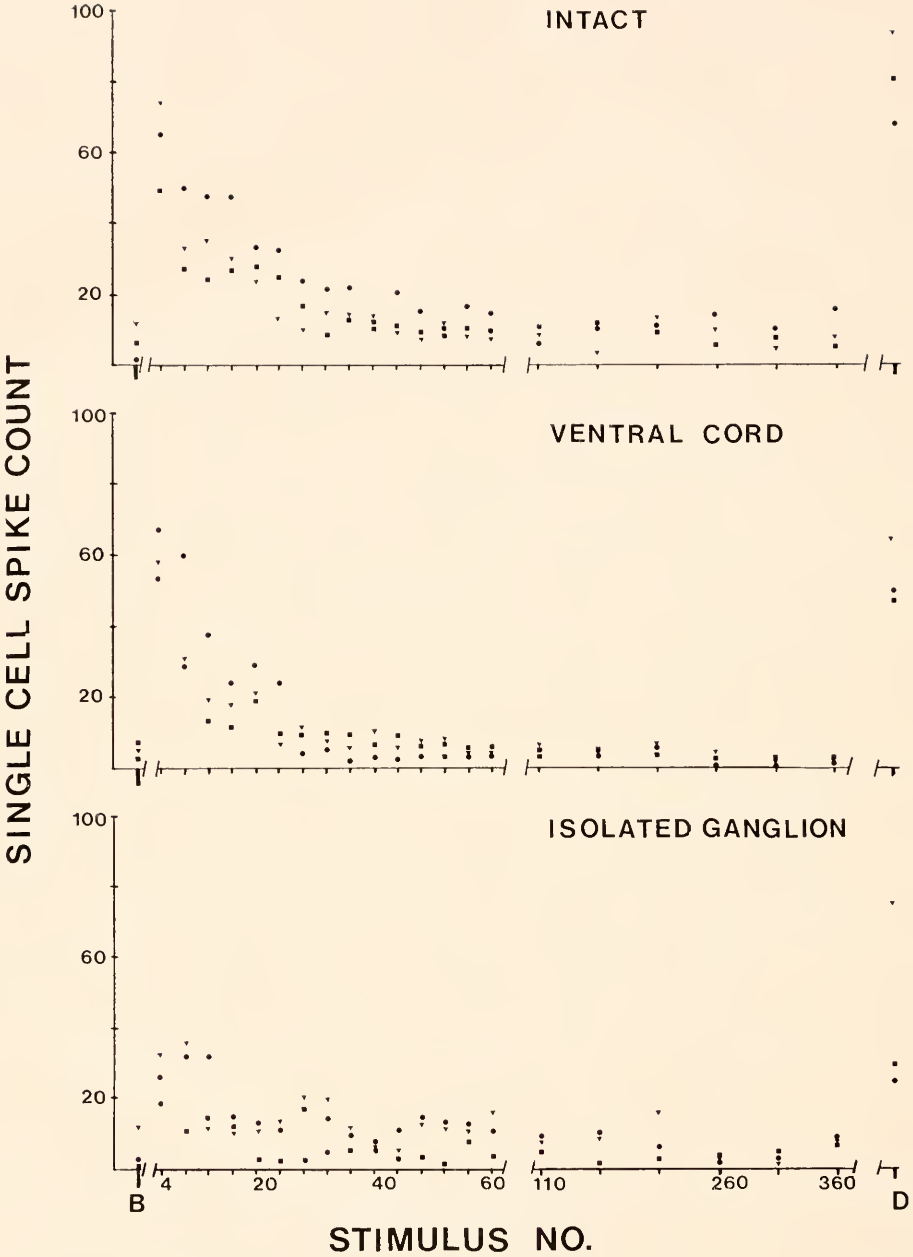

Figure 3. Examples of spike counts in single, identifiable dorsal nerve units in 3 different preparations (Intact, Ventral Cord and Isolated Ganglion) during acquisition and subsequent retests. These counts are uncorrected with respect to baseline changes. This correction amplifies differences between the acquisition and subsequent retest stages. The circle denotes acquisition; the square, 3-min retest; and the inverted triangle, 9-min retest.

data are represented as per cent values; the initial (and maximal) spike count recorded during the first air puff was used as the reference point (100%). It can be seen that in all three groups the repeated presentation of air puffs resulted in a decay of unit activity. Statistical tests indicate no significant difference between groups during the acquisition phase. A summary of the spike counts and the results of the statistical analyses for the first 60 trials are presented in Table I. After this initial and rapid response decrement, the counts in all groups leveled off and remained low for the remaining 300 stimulations (Fig. 2).

In Figure 3 we have included examples of uncorrected spike counts obtained in single, identifiable dorsal nerve units. Each of the 3 preparations is represented. These examples are preparations demonstrating moderate differences between the spike counts obtained during the initial acquisition phase and those obtained during the subsequent retest. The lower count observed in the isolated ganglion example of Figure 3 was typical in preparations of this group.

Measures of "retention"

After a 3-min respite the preparations in each group were presented another 360 stimulations to determine whether there was any persistence or retention of the response diminution over time. In all three groups there were a significantly fewer number of spikes during the first 60 trials of the 3-min retest when compared to the counts observed during the initial acquisition. In Figure 1, examination of the 3-min retest phase indicates that the initial responses of the groups were lower, and that the slopes were steeper. These lower response levels were statistically significant for the "Intact" group ($P < 0.01$), the "Ventral Cord" group ($P < 0.01$), and the "Isolated Ganglion" animals ($P < 0.05$). The individual subject totals in Table I show that for the Intact and Ventral Cord groups, 28 out of 29 subjects displayed a lower spike count during the 3-min retest. The persistance of a response decrement was not as great for the "Isolated Ganglion" preparations. Statistical analyses showed that both the Intact and the Ventral Cord groups were significantly less than the Isolated Ganglion group during the 3-min retest ($P < 0.05$ and $P < 0.02$, respectively). As in the acquisition phase, the response levels of these groups remained low for the remaining 300 stimulations.

A period of 9 minutes was allowed to elapse after completion of the 3-min retest before the third series of 360 air puffs was delivered. This 9-min retest provided information concerning a relatively long-term persistence of the response decrements and a general estimate of recovery time. In all groups there was an increase in the spike counts during the first 60 trials, but this was not significant (Fig. 1, Table I). Comparisons of the counts obtained during acquisition and those obtained during the first 60 trials of the 9-min retest yielded significantly lower scores for the Intact and Ventral Cord groups ($P < 0.02$; $P < 0.05$), but the Isolated Ganglion counts were returning to levels observed during acquisition.

"B" indicates the pre-stimulus spontaneous discharge rate; "D" indicates the application of a dishabitatuating stimulus. These examples are preparations showing moderate differences between the initial habituation and the retests.

Table 1

Data summary and statistical analysis for the first 60 puffs

Subject Numbers		Acquisition		3-minute retest		9-minute retest		Comparisons*	*P*
Intact	1	A	154	B	05	C	37	A–B	0.01
	2		108		57		66		
	3		89		60		114		
	4		65		21		55		
	5		65		28		17		
	6		72		09		16		
	7		47		07		16	A–C	0.02
	8		48		04		37		
	9		18		02		15		
	10		103		74		54		
	11		101		46		16	B–C	N.S.
	12		90		88		37		
	13		293		240		102		
	14		84		39		93		
Total Spikes			1272		680		775		
Ventral cord	1	D	89	E	56	F	71		
	2		45		16		90		
	3		216		86		55		
	4		327		126		99	D–E	0.01
	5		289		01		00		
	6		59		15		20		
	7		08		10		07		
	8		84		49		38	D–F	0.05
	9		95		39		27		
	10		229		93		231		
	11		39		14		57		
	12		65		53		65	E-F	N.S.
	13		90		18		87		
	14		33		33		73		
	15		290		23		60		
Total spikes			1958		632		980		
Isolated gangion	1	G	18	H	72	I	25		
	2		50		26		49		
	3		44		08		01	G–H	0.05
	4		106		72		26		
	5		37		27		15		
	6		71		−19		06		
	7		37		46		27	G–I	N.S.
	8		42		19		28		
	9		86		75		99		
	10		100		35		98		
	11		75		106		117	H–I	N.S.
	12		69		38		57		
	13		278		73		133		
	14		11		06		12		
Total spikes			1024		584		693		

	Comparisons**	*P*	Comparisons**	*P*	Comparisons**	*P*
	A–D	N.S.	B–E	N.S.	C–F	N.S.
	A–G	N.S.	B–H	0.05	C–I	N.S.
	D–G	N.S.	E–H	0.02	F–I	N.S.

Comparisons of the three groups during the three phases of experimentation can be made from the data presented in Figure 2. In general, it appears that all groups were equal with respect to the initial acquisition of the habituation, but that the Isolated Ganglion group demonstrated less retention during the first

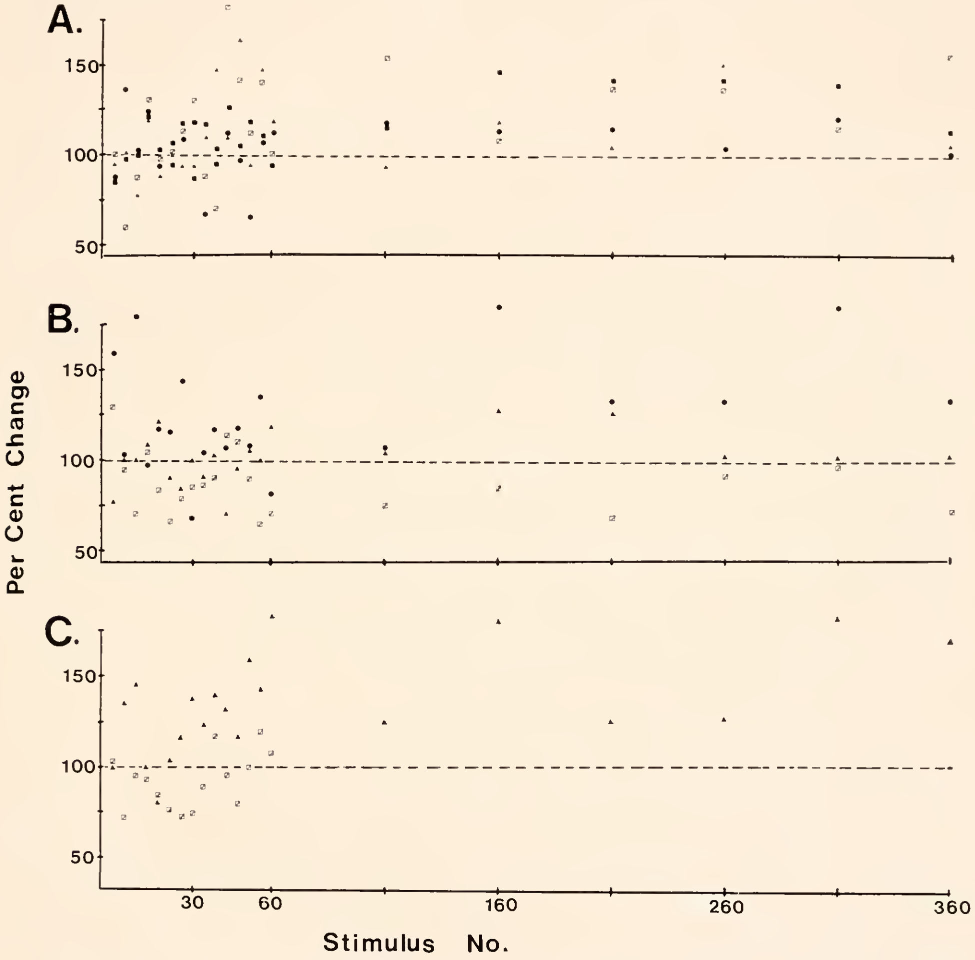

FIGURE 4. Examples of sensory nerve (ventral root) responses to air puffs. Each character (triangle, circle, etc.) represents the data obtained from a single subject. Each point is based upon an average count obtained over 4 stimulations. A, B, and C represent three blocks of 360 stimulations each, with rest periods of 3-min. and 9-min. between A-B and B-C, respectively. The initial response of the sensory nerve during the first air puff was used as the 100% reference point.

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* Wilcoxon Matched-Pairs Signed-Ranks (Siegel, 1956).
** Mann-Whitney U-Test (Siegel, 1956).
All tests are two-tailed.

60 stimulations in the 3 and 9-min retention tests. There were no differences between preparations with respect to the number of neuromeres between the stimulated gill book and the recorded ganglion dorsal nerve output. Stimulation and recording at the same segment or across two or more segments led to similar response changes during acquisition and during the two retests. Accordingly, the data of these preparations were combined within the Intact and Ventral Cord groups.

Measures of "dishabituation"

A critical control for fatigue in demonstrations of habituation is to present a stimulus of different modality or intensity. A "dishabituation" or response recovery is typically obtained. In Figure 2 the results of such tests are represented. The application of water drops, tactile stimulation with a probe, or a more intense air puff in the spot that had been receiving repetitive air puffs, produced dorsal nerve responses that equalled the spike counts obtained when these nerves were first stimulated. This demonstration of response recovery rules against fatigue in dorsal nerve output or intra-ganglionic processes as an explanation for the response decrement.

Sensory nerve responses

Recordings made from fiber branches mediating tactile input show that the repetitive stimulation of the gill book surface with air puffs did not result in any drop in the spike counts elicited by each puff (Fig. 3). If anything, the spike counts obtained from four preparations show a slight increase over the 360 stimulations. Continuation of the stimulation in retests of some of these preparations failed to demonstrate any diminution in sensory nerve response.

Discussion

Although habituation is observed in a wide variety of vertebrate and invertebrate species, certain general characteristics are common. Primarily, there is a rapid response decrement to a repetitively applied stimulus, a decrement that persists for a time, followed by a return to the original response tendencies or what is referred to as "spontaneous recovery." The post-habituation recovery emphasizes the relatively labile nature of this type of system adaptation. However, some portion of the habituation process does have a more stabile character. Frequently, a "potentiation of habituation" is observed where, after repeated habituation and spontaneous recovery cycles, the rate of habituation increases while the initial response levels may remain high. These various characteristics are usually distinguished from fatigue and sensory adaptation by two procedures. First, the application of a stimulus of a different type or intensity produces "dishabituation" or an immediate recovery of the response, thus ruling out fatigue. Secondly, analyses of afferent systems indicate that the response decrements are not due to a failure in sensory transmission. The above criteria, discussed by Thompson and Spencer (1966), have been met in the present investigations with *Limulus* abdominal ganglia. With repetitive tactile stimulation there is a rapid and persisting drop in dorsal nerve unit activity; subsequent retests indicate a potentiation of habituation for at least 9 minutes. After habituation the nerve is still responsive to a different stimulus, indicating dishabituation; the sensory nerves do not show a decay in activation with repetitive tactile stimulation. Habituation then, is demonstrated at the neuronal level in what is considered to be a living fossil.

A similar demonstration of habituation in the abdominal ganglion of an arthropod was performed by Pumphery and Rawdon-Smith (1937) in the cockroach. Repetitive stimulation of the cercus at particular frequencies resulted in what was termed "adaptation" of the ganglionic response recorded in the post-synaptic nerves, while the preganglionic afferent responses remained constant. The interpolation of one extra pulse in the stimulus train resulted in dishabituation—the post-synaptic response returned to initial levels. This degree of stimulus-specificity is unusual. Additional electrophysiological evidence for habituation in the isolated abdominal ganglion of the cockroach is reported by Hughes (1965). Stimulation of the annal cerci with air puffs at 1/sec resulted in a diminution of the giant fiber discharge with the major decay in responsivity occurring during the first 15 secs of stimulation. Recordings from the cercal nerve showed a constant rate of sensory input. A series of retests indicated some retention over time. Evidence for long-term retention of habituation in arthropods was obtained by Rowell (1968), who studied response decrements of units in the tritocerebrum of locusts. Successive presentation of stimuli (a moving black disc) was followed by a drop in unit discharge. After a 5-min interval, the stimulus series was presented again, and a more rapid decay in unit activity took place. After 4 such series the animal was allowed a 2-hour rest, and in the subsequent series the units still demonstrated an initially lower response level and more rapid habituation. In *Limulus* abdominal ganglia, a 9-min retention span was observed in the intact system and intact ventral cord region. The isolated ganglion showed little carry-over of the previous habituation in the second (9-min) retest, although there was significant retention in the 3-min retest.

Comparisons between the major groups suggest no relationship between neural complexity and initial acquisition. All groups demonstrated similar decays in unit activity during the first 360 presentations. There is, however, some relationship between neural complexity and the persistence or retention of the response decrement in the retests. While the intact and isolated cord groups displayed a persistence effect on the 9-min retest, the isolated ganglion group had almost returned to levels observed in the initial acquisition series. The response decay during the 9-min retest was similar to that recorded in the initial series of stimulus presentation. This loss of the response decrement may well be due to a metabolic deterioration. In our previous work on cardioregulatory properties of the abdominal ganglia (Corning and Von Burg, 1968; Von Burg and Corning, 1970), we had frequently noted that the experimental "lifetime" of a ganglion was shortened when it was isolated from the system. This possible deterioration does not affect acquisition, since habituation still occurred during the 9-min retest in the Isolated Ganglion group. It may well be, as Applewhite and Gardner (1971) have suggested, that acquisition of habituation is metabolically independent. It was found, for example, that the rate of habituation was independent of temperature and unaffected by inhibition of protein and RNA synthesis, while response recovery was apparently metabolically dependent. However, the work of Applewhite was on a protozoan, and the mechanisms involved during the acquisition phase may not be the same for the arthropod. Such hypotheses concerning the physiological and biochemical mechanisms underlying habituation could be tested in the abdominal ganglion preparation of *Limulus*. The arterial sheath surrounding the cord permits relatively easy and localized perfusion (Corning and Von Burg, 1970), and the persistence of a response decrement for 9-min is sufficient time for the temperature of the system to be altered or for the perfusion of agents that might disrupt or enhance the persistence of a response decrement.

SUMMARY

1. Extracellular recordings were made from dorsal root units in the abdominal ganglia of *Limulus*. These units are activated by tactile stimulation of the gill book covers.

2. The response in these units to repeated puffs of air directed on a gill book is a rapid decline in activity. The response "spontaneously recovers" with time when the stimulus is withheld. Stronger stimuli or different stimuli (water drops) produce an immediate recovery of the diminished responsivity; this recovery is similar to "dishabituation" noted in other studies and rules out fatigue as an explanation for the dorsal root response decrement. Sensory fatigue or adaptation are ruled out by the demonstration that repeated puffs of air do not produce any response decrement in the nerves (ventral roots) which carry the afferent tactile information.

3. The relationship between the number of ganglia and the acquisition of habituation was examined and no differences were found. However, during retests the Intact and Isolated Ventral Cord preparations demonstrated a better persistence of the response decrement.

4. These findings are discussed with respect to habituation data obtained by others in invertebrates.

LITERATURE CITED

Applewhite, P., and F. T. Gardner, 1971. A theory of protozoan habituation learning. *Nature*, in press.

Applewhite, P., F. T. Gardner and E. Lapan, 1969. Physiology of habituation learning in a protozoan. *Trans. New York Acad. Sciences*, **31**: 842–849.

Bullock, T. H., and G. C. Quarton, 1966. Simple systems for the study of learning mechanisms. *Neuro-Sciences Res. Program Bull., 1968*, **4**: 105–233.

Corning, W. C., and R. Von Burg, 1968. Behavioural and neurophysiological studies of *Limulus polyphemus*. In: J. Salanki Ed., *Invertebrate Neurobiology*. Plenum Press, New York, 501 pp.

Corning, W. C., R. Von Burg and R. Lahue, 1970. Endogenous and exogenous influences on *Limulus* heart rhythm. *Amer. Zool.*, **10**: 200.

Hughes, G. M., 1965. Neuronal pathways in the insect central nervous system. Pages 79–112 in J. E. Treherne and J. W. L. Beament, Eds., *The Physiology of the Insect Central Nervous System*. Academic Press, New York, 277 pp.

Hyde, I., 1893. The nervous mechanism of respiratory movements in *Limulus polyphemus*. *J. Morphol*, **9**: 431–448.

Makous, W. L., 1969. Conditioning in the horseshoe crab. *Psychonomic Sci.*, **14**: 4–6.

McConnell, J. V., 1966. Comparative physiology: learning in invertebrates. *Ann. Rev. Physiol.*, **28**: 107–136.

Ortega, J. K. E., and R. I. Gamow, 1970. *Phycomyces*: Habituation of the light growth response. *Science*, **168**: 1374–1375.

Patten, W., and W. A. Redenbaugh, 1900. Studies on *Limulus*. II. The nervous system of *Limulus polyphemus* with observations upon the general anatomy. *J. Morphol.*, **16**: 91–200.

Pumphery, R. J., and A. F. Rawdon-Smith, 1937. Synaptic transmission of nerve impulses through the last abdominal ganglion of the cockroach. *Proc. Roy. Soc. London Series B.*, **122**: 106–118.

Rowell, C. H. F., 1968. The control of reflex responsiveness and the integration of behaviour. Pages 157–164 in J. E. Treherne and J. W. L., Beament, Eds., *The Physiology of the Insect Central Nervous System.* Academic Press, New York.

Rushforth, N. B., 1967. Chemical and physical factors affecting behavior in *Hydra*: interactions among factors affecting behavior in *Hydra.* Pages 369–390 in W. C. Corning and S. C. Ratner, Eds., *Chemistry of Learning.* Plenum Press, New York.

Sharpless, S., and H. Jasper, 1965. Habituation of the arousal reaction. *Brain,* **79**: 655–682.

Siegel, S., 1956. *Nonparametric Statistics.* McGraw-Hill Inc., New York, 312 pp.

Smith, J. C., and H. D. Baker, 1960. Conditioning in the horseshoe crab. *J. Comp. Physiol. Psychol.*, **53**: 279–281.

Thompson, R. F., and W. A. Spencer, 1966. Habituation: a model phenomenon for the study of the neuronal substrates of behavior. *Psych. Rev.*, **173**: 16–43.

Thorpe, W. H., 1966. *Learning and Instinct in Animals.* Harvard University Press, Cambridge, 558 pp.

Thorpe, W. H., and D. Davenport, Eds., 1965. *Learning and Associated Phenomena in Invertebrates.* Bailliere, Tindall and Cassell, London, 190 pp.

Treherne, J. E., and J. W. L. Beament, Eds., 1965. *The Physiology of the Insect Central Nervous System.* Academic Press, New York.

Von Burg, R., and W. C. Corning, 1969. Cardioinhibitor nerves in *Limulus. Can. J. Zool.*, **47**: 735–737.

Von Burg, R., and W. C. Corning, 1970. Cardioregulatory properties of the abdominal ganglia in *Limulus. Can. J. Physiol. Pharmacol.*, **48**: 333–341.

Wasserman, G. S., and D. G. Patten, 1969. Avoidance conditioning in *Limulus. Psychonomic Sci.*, **15**: 143–144.

Westerman, R. A., 1963. A study of the habituation of responses to light in the planarian *Dugesia dorotocephala. Worm Runner's Digest,* **5**: 6–11.

Wood, D. C., 1970. Parametric studies of the response decrement produced by mechanical stimuli in the protozoan, *Stentor coeruleus. J. Neurobiology*, **1**: 345–360.

Reference: *Biol. Bull.*, **140**: 440–460. (June, 1971)

THE MARINE ENCHYTRAEIDAE (ANNELIDA, OLIGOCHAETA) OF THE EASTERN COAST OF NORTH AMERICA WITH NOTES ON THEIR GEOGRAPHICAL DISTRIBUTION AND HABITAT[1]

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The Enchytraeidae, a reputedly difficult family of Annelida Oligochaeta from a taxonomic standpoint, include marine representatives. Many genera and species are wide spread in their distribution.

In spite of the fact that many species occur in great abundance along the Atlantic coast of North America, they have received scant attention from workers. Only eight papers dealing with marine Enchytraeidae have appeared in North America. From the Atlantic coast, Minor (1863) gave a description of *Enchytraeus triventralopectinatus,* found near the high-water mark at New Haven, Connecticut. This species seems to be the naidid *Paranais litoralis* Müller, as supposed by Michaelsen (1900). Verrill (1873) described *Halodrilus littoralis* but its synonymy with *Enchytraeus albidus* Henle was later established by Michaelsen (1900); Verrill (1873, page 623) reported it from New Haven, Woods Hole and Casco Bay, Maine, as "very common under dead sea-weeds and stones near high-water mark." Smith (1895) made an anatomical study of this oligochaete at Woods Hole, using the name of *Enchytraeus littoralis* Verrill. Moore (1905) reported two species, *Enchytraeus albidus* and *Lumbricillus agilis,* probably a synonym of *Lumbricillus lineatus* (Müller), to be very abundant at Woods Hole, Vineyard Sound, Massachusetts, as well as along the coast from Casco Bay, Maine, to Sea Isle City, New Jersey. Welch (1917) carried out some ecological observations and experiments in methods of culture with *Enchytraeus albidus* and *Lumbricillus lineatus* at Woods Hole. Recently Kennedy (1966) described a new species referred to the genus *Grania* Southern 1913, from marine localities off the coast of Bimini and Panama City, Florida.

Eisen (1904), a pioneer in enchytraeid taxonomy, described eleven new marine species which are apparently limited to the Pacific west coast of North America. The descriptions were based principally on Enchytraeidae collected by the Harriman expedition to Alaska in 1899. The general procedure observed in modern descriptions of Enchytraeidae, renders indeterminable many species described before 1930. Eisen's species need critical revision; however, with type series for comparison, most of them, carefully described, would probably survive. More recently Altman (1931) described *Enchytraeus pugetensis* from Puget Sound, Washington; this species was included in the genus *Marionina* Michaelsen by

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Nielsen and Christensen (1959); due to the poor quality of the description this species in indeterminable.

During the spring 1969, the author carried out some studies on the systematics and ecology of marine Enchytraeidae living in Massachusetts and North Carolina, limited largely to salt marshes and intertidal areas in the vicinities of Woods Hole and Beaufort. Material from a survey of the fauna of Cape Cod Bay, Massachusetts, which is conducted by the Biotic Census of the Systematics-Ecology Program, Marine Biological Laboratory, was found to contain three abundant species, two of these new to science. The ecological interest of the study was suggested by the unique locations of Massachusetts and North Carolina, since the environs of Woods Hole and Cape Cod Bay have a cold environment in the boreal biogeographic province, in contrast to the Carolinian province which displays boreal and temperate features. The principal results suggest interesting problems of distribution and tolerance.

METHODS

Marine Enchytraeidae were identified from 200 samples collected by the author, in the spring of 1969 at 20 stations in the environs of Woods Hole, Massachusetts and Beaufort, North Carolina. One part of each sample was preserved in a solution of 10% formalin in seawater. The living animals in the non-preserved samples were extracted qualitatively by simple washing and filtering on nylon screens of 0.1 mm to 0.5 mm mesh diameter. The formalin-preserved animals were quantitatively extracted by washing the whole sample through a series of screens down to 0.5 mm mesh diameter. The formalin-preserved animals were separated from the residue under slight magnification, briefly immersed in a solution of 10% formalin in seawater and stored in 85% ethyl alcohol (Lasserre, 1967a, Hulings and Gray, 1971). Nineteen quantitative samples from Cape Cod Bay, Massachusetts contained enchytraeids. The samples were taken with a $0.1\ m^2$ Smith-McIntyre grab. Material was washed through a series of screens down to 0.5 mm mesh diameter, narcotized in a solution of 0.015% propylene phenoxytol, fixed for 48 hours in a solution of 10% formalin in seawater, then stored in 85% ethyl alcohol.

As a general rule only mature enchytraeids were examined. Their anatomy was observed by one, or a combination of three methods: (a) living worms were placed on a slide with a drop of water and covered with a cover-slip; the size and weight of the cover slip and the amount of water barely allow the worm restricted movements; (b) the fixed worm was lightly stained in Borax carmine of Ehrlich's acetic haematoxylin (Pantin, 1962) and mounted whole in Canada balsam; (c) the genital regions were sectioned at $5\ \mu$ and stained in Heidenhain's haematoxylin and eosin (Pantin, 1962).

The sediment was analyzed for particle size through a nest of U. S. Standard Sieves, each separated by one phi-unit. The sieves used had mesh openings of 4.000 mm, 2.000 mm, 1.000 mm, 0.500 mm, 0.250 mm, 0.063 mm. In the environmental data "Gravel" refers to particles larger than 2.000 mm, and "silt-clay" to particles smaller than 0.063 mm; "So" is the sorting coefficient, $So = \sqrt{Q1/Q3}$ (Trask, 1932), where Q1 and Q3 are, respectively, the first and the third quartiles, plotted on the cumulative curves. A well-sorted sediment has a sorting index ranging from 2.30 to 1.40; a very well-sorted sediment has a sorting index ranging from 1.40 to 0.50.

Environmental data for the principal stations are given below.

Massachusetts

Station 1: 41°32.30′N, 70°38.10′W; Falmouth Harbor beach. Salt marsh, mean particle size 0.400 mm, So = 1.29. Gravel 35.40%, sand 64.38%, silt-clay 0.22%.

Station 2: 41°31.00′N, 74°40.00′W; Nobska Beach, Woods Hole. Intertidal, semi-protected, high tide mark, depth in substratum 0.20 m; mean particle size 0.900 mm, So = 1.63. Gravel 19.20%, sand 80.78%, silt-clay 0.02%.

Station 3: 41°32.00′N, 70°40.00′W; Gansett Beach, Woods Hole. Intertidal, semi-protected, high tide mark, depth in substratum 0.10 m; mean particle size 0.500 mm, So = 1.46. Gravel 35.22%, sand 64.71%, silt-clay 0.07%.

Station 4: 41°35.00′N, 70°39.00′W; Wood Neck Beach; Sippowisset. Intertidal, semi-protected, mean high tide mark, depth in substratum 0.20 m; mean particle size 0.400 mm, So = 1.69. Gravel 15.00%, sand 84.80%, silt-clay 0.20%.

Station 5: 41°31.41′N, 70°40.41′W; Crane's Beach, Woods Hole. Intertidal, semi-protected, mean high tide mark, depth in substratum 0.10 m; mean particle size 0.500 mm, So = 1.18. Gravel 35.71%, sand 64.28%, silt-clay 0.01%.

Station 6: 41°32.00′N, 70°41.00′W; M. B. L. Beach, Woods Hole. Intertidal, semi-protected, mean high tide mark, depth in substratum 0.10 m; mean particle size 0.720 mm, So = 1.40. Gravel 33.20%, sand 66.70%, silt-clay 0.10%.

Stations of the Systematics-Ecology Program's Biotic Census, in Cape Cod Bay

0612-E1: 42°02.30′N, 70°08.00′W; depth 10.98 m; mean particle size 0.880 mm, So = 0.39. Gravel 2.10%, sand 96.20%, silt-clay 1.70%.

1412-E1: 41°54.30′N, 70°08.00′W, depth 15.55 mm; mean particle size 1.490 mm, So = 0.54. Gravel 28.03%, sand 71.51%, silt-clay 0.45%.

1412-E4: 41°54.00′N, 70°08.40′W; depth 17.08 m; mean particle size 0.795 mm, So = 0.52. Gravel 2.69%, sand 97.27%, silt-clay 0.01%.

1530-E3: 41°53.00′N, 70°31.20′W; depth 12.20 m; mean particle size 0.336 mm, So = 0.68. Gravel 0%, sand 99.98%, silt-clay 0.01%.

1730-E3: 41°51.00′N, 70°31.20′W; depth 8.54 m; mean particle size 0.576 mm, So= 0.64. Gravel 0%, sand 99.99%, silt-clay 0%.

1812-E1: 41°50.30′N, 70°08.00′W; depth 2.74 mm; mean particle size 0.552 mm, So = 0.61. Gravel 0%, sand 98.99%, silt-clay 0.99%.

1812-E2: 41°51.00′N, 70°07.10′W; depth 2.13 m; mean particle size 0.702 mm, So = 0.50. Gravel 2.28%, sand 97.71%, silt-clay 0%.

1812-E4: 41°50.00′N, 70°08.50′W; depth 4.27 m; mean particle size 0.402 mm, So = 0.66. Gravel 0.06%, sand 99.92%, silt-clay 0%.

1812-E5; 41°51.00′N, 70°08.50′W; depth 3.05 m; mean particle size 0.461 mm, So = 0.64. Gravel 0%, sand 99.98%, silt-clay 0%.

1910-E1: 41°49.30′N, 70°05.30′W; depth 6.71 m; mean particle size 1.021 mm, So = 0.41. Gravel 24.32%, sand 74.84%, silt-clay 0.82%.

1910-E2: 41°50.00′N, 70°04.40′W; depth 6.71 m; mean particle size 0.868 mm, So = 0.51. Gravel 4.48%, sand 95.48%, silt-clay 0.01%.

1930-E2: 41°50.00′N, 70°31.20′W; depth 10.37 m; mean particle size 0.694 mm, So = 0.65. Gravel 0.16%, sand 99.82%, silt-clay 0%.

1930-E3: 41°49.00′N, 70°31.20′W; depth 11.89 m; mean particle size 0.698 mm. So = 0.64. Gravel 0.44%, sand 99.54%, silt-clay 0%.

2008-E5: 41°49.00′N, 70°03.15′W.

2028-E5: 41°49.00′N, 70°30.00′W; depth 18.80 m.

2130-E2: 41°48.00′N, 70°31.20′W; depth 6.71 m, mean particle size 0.466 mm, So = 0.58. Gravel 0.06%, sand 98.86%, silt-clay 1.07%.

2318-E1: 41°45.30′N, 70°16.00′W; depth 14.64 m, mean particle size 0.740 mm, So = 0.52. Gravel 5.51%, sand 93.39%, silt-clay 1.07%.

2318-E4: 41°45.00′N, 70°16.40′W; depth 14.94 m, mean particle size 0.674 mm, So = 0.23. Gravel 36.25%, sand 61.83%, silt-clay 1.86%.

North Carolina

Station 1: 34°44.40′N, 76°39.40′W; Newport River, North Gallant Point. Salt marsh; mean particle size 0.300 mm, So = 1.79. Gravel 0%, sand 99.98%, silt-clay 0.02%.

Station 2. 34°45.40′N, 76°44.10′W; Newport River, Lawton Point. Salt marsh; mean particle size 0.350 mm, So = 1.29. Gravel 0%, sand 99.97%, silt-clay 0.03%.

Station 3: 34°43.30′N, 76°55.30′W; west of Morehead City. Intertidal, semi-protected, mean high tide mark, depth in substratum 0.20 m; mean particle size 0.440 mm, So = 1.59. Gravel 0%, sand 99.98%, silt-clay 0.02%.

Station 4: 34°42.43′N, 76°40.55′W; Radio Island, Beaufort. Intertidal, semi-protected, mean high tide mark, depth in substratum 0.20 m; mean particle size 0.350 mm, So = 1.80. Gravel 1.00%, sand 98.64%; silt-clay 0.36%.

Station 5: 34°41.55′N, 76°35.00′W; west of Harkers Island. Intertidal, semi-protected, mean high tide mark, depth in substratum 0.20 m; mean particle size 0.360 mm, So = 1.30. Gravel 0%, sand 99.98%, silt-clay 0.02%.

Station 6: 34°41.10′N, 76°31.45′W; Harkers Island, Shell Point. Intertidal, exposed, mean high tide mark, depth in substratum 0.30 m; mean particle size 0.400 mm, So = 1.73. Gravel 0%, sand 99.95%, silt-clay 0.06%.

Station 7: 34°40.50′N, 76°55.34′W; Bogue Banks. Intertidal, exposed strand, mean high water mark, depth in substratum 0.30 m; mean particle size 0.350 mm, So = 1.14. Gravel 0%, sand 99.98%, silt-clay 0.02%.

Station 8: 34°41.50′N, 76°44.04′W; Bogue Banks, Atlantic Beach. Intertidal, exposed strand, mean high tide mark, depth in substratum 0.30 m; mean particle size 0.250 mm. So = 1.61. Gravel 0%, sand 99.95%, silt-clay 0.05%.

Station 9: 34°41.36′N, 76°41.12′W; Bogue Banks, Fort Macon. Intertidal, exposed strand, mean high tide mark, depth in substratum 0.30 m; mean particle size 0.350 mm, So = 1.87. Gravel 0.50%, sand 99.43%, silt-clay 0.07%.

Station 10: 34°40.40′N, 76°36.50′W; Schakleford Banks, facing Back Sound. Intertidal, semi-protected, mean high tide mark, depth in substratum, 0.30 m; mean particle size 0.450 mm, So = 1.19. Gravel 0%, sand 99.87%, silt-clay 0.13%.

Station 11: 34°40.25′N, 76°37.00′W; Schakleford Banks, facing open Ocean. Intertidal, exposed strand, mean high tide mark, depth in substratum 0.40 m; mean particle size 0.500 mm, So = 2.20. Gravel 0%, sand 99.99%, silt-clay 0.01%.

Station 12: 34°38.37′N, 76°30.52′W; Core Banks, lighthouse Bay, facing Core Sound. Intertidal, semi-protected, mean high tide mark, depth in substratum 0.20 m; mean particle size 0.340 mm, So = 1.25. Gravel 0%, sand 99.82%, silt-clay 0.08%.

Station 13: 34°38.28′N, 76°30.36′W; Core Banks, facing open Ocean. Intertidal, exposed strand, mean high tide mark, depth in substratum 0.40 m; mean particle size 0.450 mm. So = 2.14. Gravel 0%, sand 99.99%, silt-clay 0.01%.

Station 14: 34°11.07′N, 76°11.06′W; off the coast, continental shelf, depth 60 m, "Amphioxus sand."

Station 15: 34°10.06′N, 76°10.00′W; off the coast, continental shelf, depth 56 m, "Amphioxus sand."

SYSTEMATIC SECTION

Henlea Michaelsen, 1889

Henlea ventriculosa (Udekem, 1854)

Enchytraeus ventriculosus Udekem, 1854, page 864, Figures 1, 4, 6–9.

Henlea ventriculosa (Udekem). Michaelsen, 1889, page 31; 1900, page 69; Ude, 1929, page 56; Bülow, 1955, page 254, plate 41, Figures 1–2; 1957, page 74; Nielsen and Christensen, 1959, page 62, figures 50, 53, 57; Cekanovskaya, 1962, page 300, Figure 189.

TYPE MATERIAL: Not designated, not located.

TYPE LOCALITY: Belgium.

DESCRIPTION: The species conforms to the original description as emended by Michaelsen (1889) and by Nielsen and Christensen (1959).

HABITAT: Salt marshes, above the upper part of the shore.

DISTRIBUTION: Cosmopolitan. Europe: Finland, Sweden, Denmark, Germany, Poland, U. S. S. R., Italy, Belgium, France, Great Britain, Eire. New Zealand. South America. New records for North America: Massachusetts; station 1, (4/3) North Carolina; station 1, (2/2) (refer to number of specimen and their maturity, for example, (4/3) and (2/2) mean: 4 specimens, 3 mature and 2 specimens, 2 mature).

Cernosvitoviella Nielsen and Christensen, 1959

Cernosvitoviella immota (Knöllner, 1935)

Enchytraeoides immotus Knöllner, 1935b, page 438, Figures 9–13; Bülow, 1955, page 255, plate 41, Figures 5–6; 1957, page 85.

Cernosvitoviella immota (Knöllner). Nielsen and Christensen, 1959, page 40. Figures 19–22.

TYPE MATERIAL: Not designated and not located.

TYPE LOCALITY: Kiel Bay, Western Germany.

DESCRIPTION: The species conforms to the original description, supplemented by Nielsen and Christensen (1959).

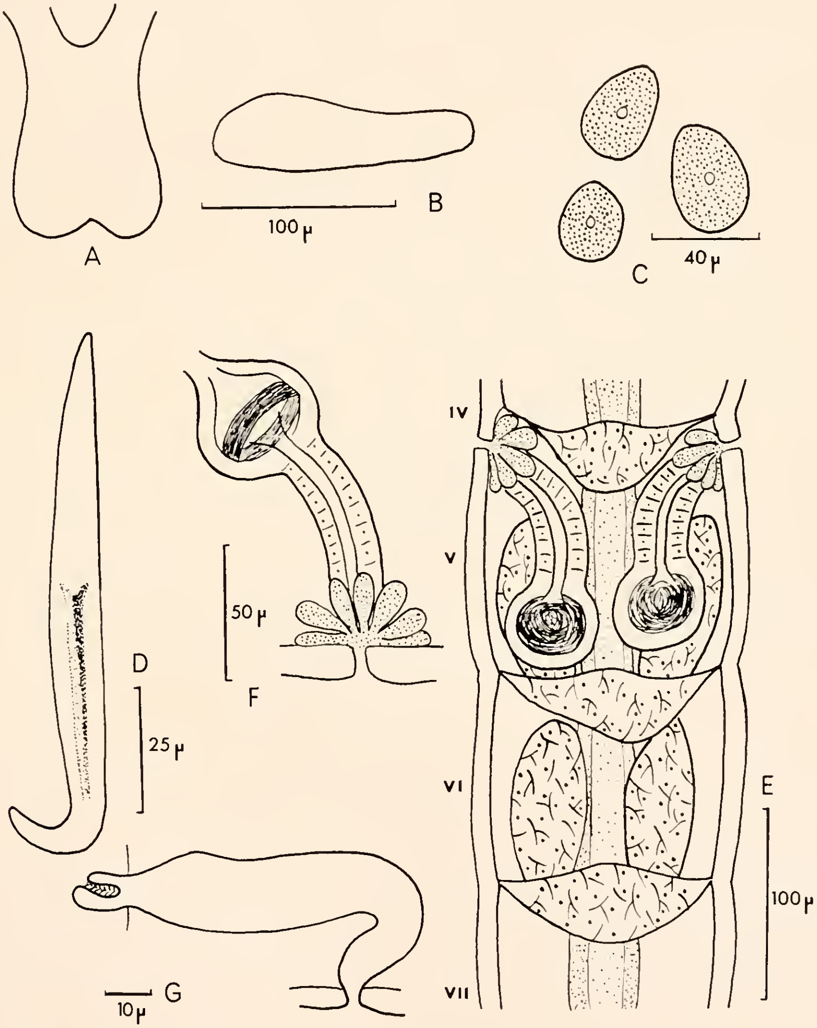

FIGURE 1. *Lumbricillus codensis* nov. sp. A, brain in dorsal view; B, brain in lateral view; C, coelomocytes; D, setae; E, dorsal view of septal glands and spermathecae (the ental ducts are not visible dorsally); F, spermatheca, detail; G, nephridium.

Habitat: Salt marshes, above upper part of the shore.

Distribution: Europe: widely distributed, Finland, Sweden, Denmark, Germany, British Isles, France. New records for North America: Massachusetts; station 1, (2/2).

Enchytraeus Henle, 1837

Enchytraeus albidus Henle, 1837

Enchytraeus albidus Henle, 1837, page 74; Michaelsen, 1900, page 89; Knöllner, 1935b, page 449; Cernosvitov, 1937, page 291; Bülow, 1957, page 91; Nielsen and Christensen, 1959; page 91, Figures 95–100; Cekanovskaya, 1962, page 307, Figure 193.

Synonyms: *Halodrilus littoralis* Verrill, 1873; *Enchytraeus littoralis* (Verrill), Smith, 1895; *Enchytraeus mobii* Michaelsen, 1886; *Enchytraeus sabulosus* Southern 1906; *Litorea krumbachi* Cejka, 1913; *Enchytraeus constrictus* Backlund, 1947 .

Type Material: not designated and not located.

Type Locality: Kiel Bay, West Germany.

Description: The species conforms to the reviewed description of Nielsen and Christensen (1959).

Habitat: Salt marshes, decaying seaweed, compost heaps, sewage beds, effluents.

Distribution: Cosmopolitan. Europe: Finland, Denmark, Sweden, British Isles, Holland, France, Greenland, South Patagonia, Uruguay, Tierra del Fuego. North America: New Haven, Woods Hole, Sea Isle City, New Jersey, Casco Bay, Maine (Verrill, 1873; Smith, 1895; Moore, 1905; Welch, 1917); Cold Spring Harbor, Long Island, New York (Hunt, 1915); Newport River, North Carolina (Wells, 1961). New records in North America: Massachusetts: station 1 (100/74); station 2, (63/52). North Carolina: station 1, (6/6); station 3, (3/3).

Enchytraeus capitatus Bülow, 1957

Enchytraeus capitatus Bülow, 1957, page 93, Figures 13–17; Nielsen and Christensen 1959, page 92; 1963a, page 14, Figure 11.

Type Material: Not designated, not located.

Type Locality: West Germany, Kimbrischen Halbinsel, among *Armeria* on dike at Büsum.

Description: The species conforms entirely to the original description, reviewed by Nielsen and Christensen (1959, 1963a).

Habitat: Driftline of seaweed deposit; intertidal sandy beaches in upper shore; meiobenthos.

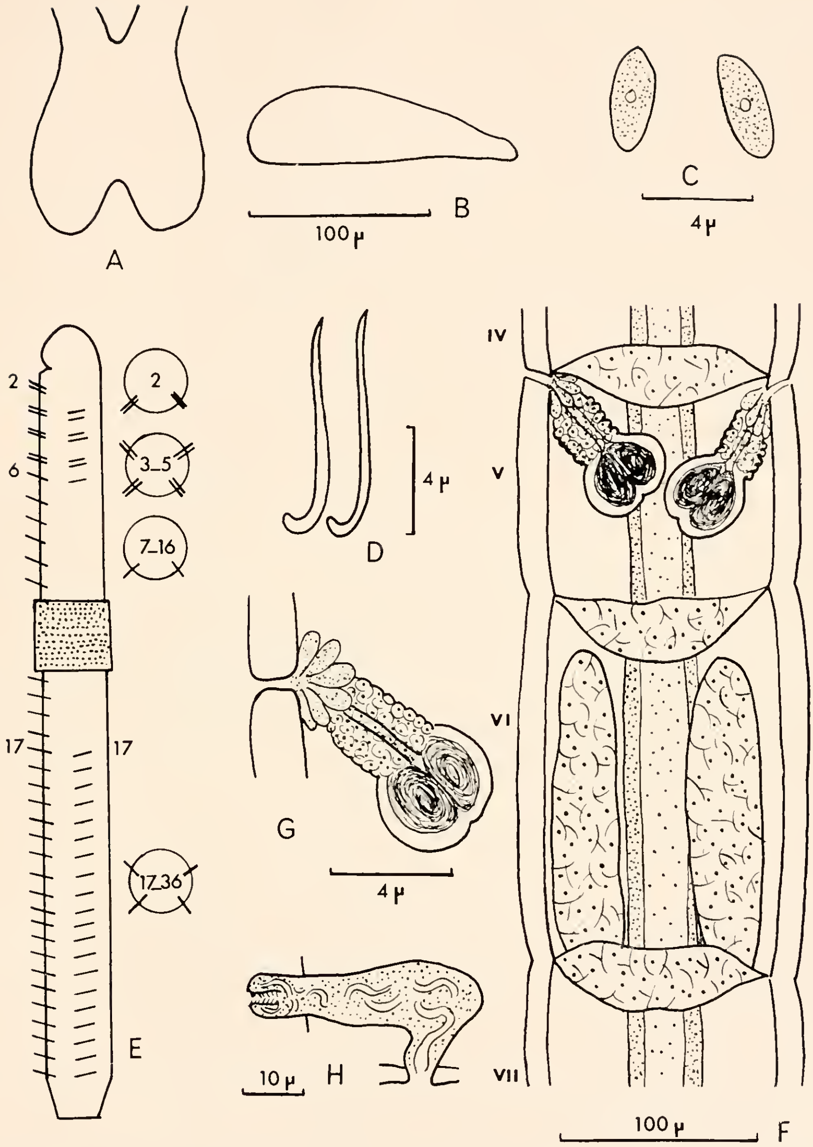
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Figure 2. *Marionina welchi* nov. sp. A, brain in dorsal view; B, brain in lateral view; C, coelomocytes; D, setae; E, complement of setae in diagrammatic view; F, dorsal view of septal glands and spermathecae (the ental ducts are not visible dorsally); G, spermatheca, detail; H, nephridium.

Distribution: Europe: Denmark, West Germany, France Atlantic and Mediterranean coasts. New records for North America. Massachusetts: station 1, (13/10); station 2 (122/100); station 3, (258/234); station 5, (36/26). North

Carolina: station 1, (6/5); station 2, (7/4); station 3 (21/17); station 5, (8/5); station 7, (5/1); station 10, (50/38).

Lumbricillus Ørsted, 1844

Lumbricillus lineatus (Müller, 1774)

Pachydrilus lineatus (Müller). Ude, 1929, page 60, Figure 76; Knöllner, 1935b, page 435; Bülow, 1955, page 254, plate 41, Figures 3–4.

Lumbricillus lineatus (Müller). Welch, 1917, page 123; Bülow, 1957, page 76; Nielsen and Christensen, 1959, page 100, Figures 109–112; Cekanovskaya, 1962, page 310, Figure 197.

Synonyms: *Lumbricillus verrucosus* (Claparède, 1864); *Lumbricillus claparedeanus* Ditlevsen, 1904; *Lumbricillus agilis* Moore, 1905

TYPE MATERIAL: not designated and not located.

TYPE LOCALITY: Denmark.

DESCRIPTION: The species conforms to the reviewed descriptions of Ude, 1929, and Nielsen and Christensen, 1959.

HABITAT: Salt marshes, in upper littoral zone; driftline seaweed deposit.

DISTRIBUTION: Europe: widely distributed. Hebrides, Tierra del Fuego. North America: Casco Bay, Maine to Vineyard Sound, Massachusetts (Moore, 1905, page 395); Woods Hole, Massachusetts (Welch, 1917, page 123). New records in North America: Massachusetts: station 2, (18/14); station 3, (15/9). Not found in North Carolina.

Lumbricillus codensis nov. sp.

Figure 1

HOLOTYPE: United States National Museum (U. S. N. M.), Cat. No. 43476. Cape Cod Bay, Massachusetts, U. S. A. 41°48.00′N, 70°31.20′W. Depth 6.7 meters.

PARATYPES: U. S. N. M. Cat. No. 43477. Five individuals as type locality; U. S. N. M. Cat. No. 43478, one individual from 41°53.00′N, 70°31.20′W, depth 12.2 meters, Gray Museum, Marine Biological Laboratory, Woods Hole, 2130, three individuals as type locality, 1930, one individual from 41°54.30′N, 70°08.00′W, depth 15.6 meters, 2130, one individual from 41°49.00′N, 70°31.20′W, depth 11.9 meters.

DERIVATION: "codensis" from Cape Cod Bay, Massachusetts, U. S. A.

DESCRIPTION: Small to medium sized, length 6–12 mm, diameter 140–160 μ. 45 to 58 segments. Color whitish. Dorsal setal bundles absent, ventral bundles present, each containing only one straight seta, strongly hooked proximally, thickest in the middle and with a longitudinal depression (Fig. 1, D). Cutaneous

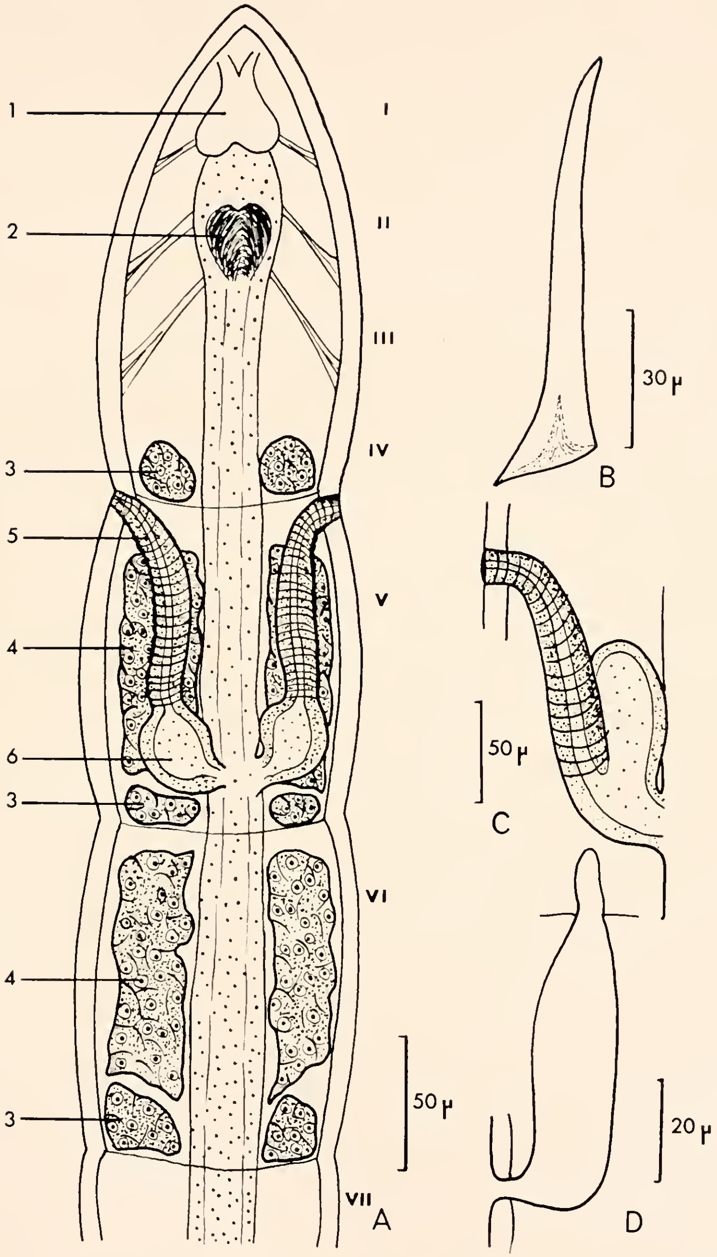
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FIGURE 3. *Hemigrania postclitellochaeta* (Knöllner, 1935). A, dorsal view of septal glands and spermathecae: 1—brain, 2—pharynx, 3—primary septal glands, 4—secondary septal glands, 5—ectal canal of spermatheca, 6—ampulla of spermatheca, B, setae; C, spermatheca, detail; D, nephridium.

glands small and irregularly scattered. Brain longer than wide, posterior margin notched (Fig. 1, A–B). Three pairs of septal glands in IV/V–VI/VII, all united mid-dorsally, those in V/VI and VI/VII with ventral lobes, those in VI/VII large (Fig. 1, E). Chloragogen cells present from VI, particularly dense in postclitellar segments, they contain refractile globules. Coelomocytes oval or pear-shaped, $\frac{1}{3}$ to $\frac{1}{2}$ length of setae, with small granules. Dorsal vessel originates in XIII or XIV, blood colorless or faintly red. Nephridia with postseptale elongate, efferent duct arising terminally, anteseptale consisting of funnel only (Fig. 1, G). Irregularly lobed seminal vesicle, may occupy X and XI. Sperm funnel up to twelve times longer than wide, length approximately two times diameter of worm, collar regular and slightly narrower than funnel body. Sperm duct very long, irregularly coiled in XII or extending as far back as XX. Penial bulb compact, medium sized. Clitellum extends over XII-$\frac{1}{2}$ XIII, prominent gland cells arranged in transverse rows. Usually only one mature egg present at a time. Spermatheca with subspherical ampulla sharply defined from both ectal and ental ducts, ental duct short, communicating with oesophagus, ectal duct 2–3 times longer than wide, ectal orifice surrounded by a ring of 10–13 large club shaped glands, ectal duct 2–3 times longer than ampulla (Fig. 1, E, F).

HABITAT: Subtidal sands: Mean particle size between 0.336 mm and 0.795 mm.

DISTRIBUTION: Known only from Cape Cod Bay, Massachusetts, U. S. A.; material received from the Systematics-Ecology Program's Biotic Census, stations: 1412E1, (1/1); 1412E4, (24/22); 1530E3, (2/2); 1730E3, (46/38); 1812E1, (127/98); 1812E2, (286/204); 1812E4,(20/17); 1812E5, (72/58); 1930E3, (3/2); 2130E2, (78/64).

REMARKS: The shape and arrangement of the setae of *L. codensis* have no equivalent in other species of the genus. The single large and straight setae in each ventral bundle resemble those of species included in the genera *Hemigrania* nov. gen. and *Grania* Southern, 1913. However, on the morphological evidence of septal glands (no secondary glands, primary glands attached dorsally), peptonephridia (absent), nephridia (anteseptale consisting of funnel only) and spermatheca, it appears that the genus *Lumbricillus* Ørsted, 1844 is more appropriate for this species.

Marionina Michaelsen, 1889

Marionina southerni (Cernosvitov, 1937)

Pachydrilus Marionina southerni Cernosvitov, 1937, page 293, *Marionina southerni* (Cernosvitov, 1937), Nielsen and Christensen, 1959, page 112, Figures 135–137; Lasserre, 1971.

SYNONYMS: *Enchytraeus lobatus* Southern, 1909; *Enchytraeus nodosus* Stephenson, 1911; *Fridericia pseudoargentea* Knöllner, 1935.

TYPE MATERIAL: not designated and not located.

TYPE LOCALITY: Ireland.

Description: The species conforms to the reviewed description of Nielsen and Christensen (1959).

Habitat: Driftline of seaweed deposit on the middle stretch of the beach.

Distribution: Europe: Sweden, Denmark, Scotland, North Wales, Ireland, Germany, France (Atlantic and Mediterranean). New records for North America. Massachusetts: station 1, (2/2); station 2, (15/11); station 4, (7/4); station 5, (8/6). North Carolina: station 3, (12/9); station 4, (4/3); station 6, (13/10); station 10, (9/8).

Marionina spicula (Leuckart, 1847)

Enchytraeus spiculus Leuckart, 1847, page 146; Ude, 1929, page 70, Figure 80; Bülow, 1957, page 92.

Marionina spicula (Leuckart), Nielsen and Christensen, 1959, page 115, Figures 145–148; 1963a, page 20; Lasserre, 1971.

Type Material: not designated and not located.

Type Locality: Germany, Helgoland.

Description: The species conforms to the reviewed description of Nielsen and Christensen (1959).

Habitat: Driftline of seaweed deposit, on the middle stretch of the beach; intertidal sandy beaches, upper to lower shore, meiobenthos.

Distribution: Europe: widely distributed, Denmark, Germany, Belgium, Poland, France Atlantic and Mediterranean, Bulgaria Black Sea. New records for North America. Massachusetts: station 1, (122/87): station 2, (118/96); station 3, (111/82); station 4, (151/101); station 5, (186/113); station 6, (102/88). North Carolina: station 1, (18/12); station 4, (22/11); station 6, (9/8): station 7, (32/27); station 8, (15/10); station 9, (13/9); station 10, (400/268); station 11, (12/8); station 12, (54/48).

Marionina subterranea (Knöllner 1935)

Michaelsena subterranea Knöllner 1935a, page 136, 1935b, page 455, Figures 26–28; Bülow, 1957 page 94; Nielsen and Christensen, 1959, page 110, Figures 132–134; 1963a, page 19; Lasserre, 1966, page 315, 1971.

Synonyms: *Michaelsena* n. sp. Bülow 1957; *Hichaelsena glandulifera* Jansson, 1961.

Type Material: not designated and not located.

Type Habitat: Kiel Bay, West Germany.

Description: The species conforms to the original description reviewed by Nielsen and Christensen (1959, 1963a).

Habitat: Intertidal sandy beaches, on lower part of the beach, at ground water level, meiobenthos.

Distribution: Europe: widely distributed, Denmark, Sweden, Finland, Germany, British Isles, France (Atlantic and Mediterranean), Bulgaria (Black sea). North Africa: Tunisia, Algeria. New records for North America. Massachusetts: station 1, (8/5); station 2, (181/139); station 3, (603/394); station 4, (242/101); station 5, (986/421); station 6 (308/116). North Carolina: station 2 (7/6); station 5, (6/4); station 6, (5/3); station 7, (2/2); station 8, (13/11); station 9, (22/15), station 10, (42/29), station 11, (7/2); station 12, (31/22); station 13, (8/5).

Marionina achaeta Lasserre, 1964

Michaelsena sp. Hagen, 1951, cited in Bülow 1957, page 95; Nielsen and Christensen, 1959, page 110.

Marionina achaeta Lasserre, 1964, page 87; 1966, page 300, Figure 1; Tynen and Nurminen, 1969, page 151, Lasserre 1971.

Type Material: Museum National d'Histoire Naturelle, Paris, Cat. No. A.777-AA 44.

Type Locality: Bassin d'Arcachon, Atlantic, France.

Description: The species conforms to the original description (Lasserre 1964, 1966). This species was discovered by Hagen (1951) in Germany, named by him *Michaelsena achaeta* n. sp., but the description was not published. The identity of the German and French material was checked by Lasserre (1964) who retained the proposed name by Hagen and gave the description.

Habitat: Intertidal sandy beaches, in upper shore and at ground water level on lower shore, meiobenthos.

Distribution: Europe: widely distributed, Sweden, Denmark, Germany, France (Atlantic and Mediterranean), Bulgaria (Black Sea). North Africa: Tunisia, Algeria. New records for North America. Massachusetts: station 2, (3/1) station 3, (28/16); station 5 (41/12); station 6 (34/18). North Carolina: station 1, (11/5); station 2, (5/3); station 3, (33/21); station 4 (17/11); station 6, (19/7); station 7, (4/1); station 8, (7/2); station 10, (12/5); station 11, 1 immature; station 12, (8/3).

Marionina preclitellochaeta Nielsen and Christensen, 1963

Marionina sp. Jansson, 1962.

Marionina preclitellochaeta Nielsen and Christensen, 1963b, page 12, Figures 18–19; Lasserre, 1966, page 314; 1967a, and b; Tynen and Nurminen, 1969, page 151; Lasserre, 1971.

Type Material: Mols Laboratory, Femmøller, Denmark.

Type Locality: Sweden, Swedish west coast.

Description: The species conforms with the original description (Nielsen and Christensen, 1963b). This species was discovered by Jansson (1962) in Sweden and named by him *Marionina preclitellochaeta* n. sp. Jansson deferred the description to a later publication. Nielsen and Christensen (1936) found the species in Denmark, the identity of the Swedish and Danish material was checked by Nielsen and Christensen who retained the proposed name and gave the description.

Habitat: Intertidal sandy beaches, middle to lower shore, at ground water level.

Distribution: Europe: Sweden, Denmark, France Atlantic. New records for North America. Massachusetts: station 1, (170/130); station 2 (218/159); station 3, (106/67; station 5, (319/202); station 6, (216/109). North Carolina: station 4, (83/71); station 8, (9/4).

Marionina welchi nov. sp.

Figure 2

Holotype: U. S. N. M. Cat. No. 43479, Cape Cod Bay, Massachusetts, U. S. A., 41°54.00′N, 70°08.40′W, depth 17.0 meters.

Paratypes: U. S. N. M. Cat. No. 43480, three individuals as type locality; U. S. N. M. Cat. No. 43481, one individual from 41°45.30′N, 70°16.00′W, depth 14.6 meters; Gray Museum at the Marine Biological Laboratory, Woods Hole, Massachusetts, 1412, one individual as type locality.

Derivation: "*welchi*" = in honor of P. S. Welch, long an outstanding American authority on the Enchytraeidae.

Description: Small to medium-sized, length 5–10 mm, diameter 100–150 μ. 30–44 segments. Color white. Straight setae with proximal hook (Fig. 2, D), dorsal setal bundles absent in segment II and VII-XVI, two dorsal setae per bundle in segment III-V, one in segment VI, and one per bundle from segment XVII; ventral bundles containing two setae in segments II–V and one from segment VI (Fig. 2, E). Cutaneous glands arranged in five to seven transverse rows on each segment. Brain longer than wide, posterior end incised (Fig. 2, A–B). Three pairs of septal glands in IV/V–VI/VII, all united middorsally, those in VI/VII with large ventral lobes (Fig. 2, F). Chloragogen cells present from V and forming a dense layer from VI, contain refractile globules. Coelomocytes spindle-shaped, $\frac{1}{2}$ length of setae, with small granules (Fig. 2, C). Dorsal vessel originates in XIV, blood colorless. Anteseptale of nephridium ovoid, with coils of nephridial canal, postseptale elongate (Fig. 2, H). Seminal vesicle compact in segment XI. Sperm funnel $2\frac{1}{2}$ times longer than wide, length approximatively half diameter of the worm, collar regular and about half as wide as funnel body. Sperm duct narrow, of medium length and irregularly coiled in XII. Penial bulb large and compact. Clitellum extends over XII-$\frac{1}{2}$ XIII, gland cells arranged in transverse rows. Only one mature egg present at a time. Spermatheca with ectal duct short and covered by fused glands closely packed, rosette of six to eight separate glands at the ectal orifice, ampulla subspherical, length approximately equal to that of ectal and connected dorsally with oesophagus through a short ental duct (Fig. 2 F–G).

HABITAT: Subtidal sands: Mean particle size between 0.466 mm and 0.795 mm.

DISTRIBUTION: Known only from Cape Cod Bay, Massachusetts, U. S. A.; material received from the Systematics-Ecology Program's Biotic Census; stations: 1412E4, (11/7); 2028E5, (4/3); 2318E1, (2/2), 2318E4, (1/1).

REMARKS: *M. welchi* is related to *M. subtilis* (Ude, 1896), *M. paucispina* (Eisen, 1904) and *M. normani* (Michaelsen, 1907) described, respectively, from Tierra del Fuego, Santa Barbara, California and Western Australia. These species form a complex of Enchytraeidae characterized by reduction in the number of dorsal and ventral setae and by the shape of the spermatheca. The original description of *M. normani* was based on immature individuals and it must be considered a species dubium.

Hemigrania nov. gen.

DEFINITION: Peptonephridia absent. Three pairs of primary septal glands in segments IV, V, VI not united dorsally, secondary glands always present. Setae large and straight, very broad at the inner end, without nodulus; only one seta per bundle. Anteseptale of nephridia consists of funnel only. Spermatheca attached dorsally to the oesophagus, ectal duct and orifice without glands or diverticulae.

TYPE SPECIES: *Hemigrania postclitellochaeta* (Knöller, 1935b) nov. comb.

Figure 3

REMARKS: The species here referred to the new genus *Hemigrania* belonged in the genus *Michaelsena* Ude, however Nielsen and Christensen (1959) recognized *Michaelsena* as being a wholly artificial genus and transferred the species *postclitellochaeta* to *Marionina* Michaelsen, 1889. Recently, Lasserre (1967a, 1967b) was able to examine living and fixed specimens of the species *postclitellochaeta* and recognized that it differed in many ways from the other species of *Marionina*. The genus *Grania* Southern, 1913 was obviously a more suitable recipient, nevertheless, the author concluded that it might become necessary to remove it to a separate genus (Lasserre, 1967a, 1967b). This course has been followed in the present work. It is evident from a study of French and North American material, that the species described by Knöllner (*postclitellochaeta*) differed consistently from species of *Grania* Southern, and from species of other genera mainly by the presence of secondary glands and absence of peptonephridia and the shape of spermatheca. These differences are of sufficient importance to transfer *postclitellochaeta* to a new genus. The same conclusion is drawn for two species described by Michaelsen (1888 and 1907) from South Georgia and South West Australia: *Enchytraeus monochaetus* Michaelsen, 1888 and *Michaelsena principissae* Michaelsen, 1907. The important character differentiating the genus *Grania* Southern, 1913, and *Hemigrania* nov. gen. is the presence or absence of peptonephridia. *Hemigrania* nov. gen. is, evidently, closely related to the genera *Grania* and *Marionina* but differs in two characteristic features, *i.e.*, absence of peptonephridia and presence of secondary septal glands. The list of characters given in the definition of *Hemigrania* nov. gen. also enables it to be distinguished from other genera of Enchytraeidae.

Hemigrania postclitellochaeta (Knöllner, 1935) nov. comb.

Michaelsena postclitellochaeta Knöllner, 1935b, page 449, Figures 19–25; Bülow, 1957, page 95.

Marionina postclitellochaeta (Knöllner), Nielsen and Christensen, 1959, page 110.

Grania postclitellochaeta (Knöllner), Lasserre 1966, pages 299, 312–314; 1967b, page 279, page 280.

Type Material: not designated and not located.

Type Locality: Kiel Bay, West Germany, off the coast.

Other Material: Museum National d'Histoire Naturelle, Paris, Cat. No. A.777-AA 48 Gulf of Gascogny, France. U. S. N. M., Cat. No. 43482, Cape Cod Bay, Massachusetts, U. S. A., three individuals from 41°54.00'N, 70°08.40'W, depth 17.1 meters; Gray Museum, Marine Biological Laboratory, Woods Hole, Massachusetts, Sep 1910/1930, two individuals from 41°49.30'N, 70°05.30'W, depth 6.9 meters, and from 41°50.00'N, 70°31.20'W, depth 10.4 meters.

Description: (from literature and author's observations). Small to medium-sized, length 5–10 mm, diameter 100–130 μ, 32–51 segments. Color whitish. Dorsal setal bundles absent, ventral setae one per bundle, absent from anterior segments 1 to XIV or XVII, setae large, straight and very broad proximally (Fig. 3, B). Cutaneous glands small and irregularly scattered. Brain about as long as broad, posterior end incised (Fig. 3 A1). Septal glands not united dorsally, three pairs of primary glands present in IV, V, VI with arrangement of secondary glands variable; usually one pair of primary glands in IV, one pair of primaries and one pair of secondaries in V and VI (Figs. 3 and 4). Chloragogen cells present from VII, with refractile globules. Coelomocytes oval abundant posteriorly, $\frac{1}{3}$ length of setae, with refractile granules. Dorsal vessel originated behind clitellum in XX, blood faintly yellow. Nephridia with postseptale elongate, efferent duct arising terminally, anteseptale consisting of funnel only (Fig. 3, D). Compact seminal vesicle present. Sperm funnel four times longer than wide, length approximately same as diameter of the worm, collar regular, nearly as wide as funnel itself. Sperm duct long, extending as far back as XVI. Penial bulb compact and large. Clitellum extends over XII-$\frac{1}{2}$ XIII, gland cells arranged in transverse rows. One mature egg present at a time. Spermatheca with ectal duct long cylindrical and muscular, devoid of glands along its length and aperture, opening dorso-laterally, ampulla globular and connected dorsally with esophagus (Fig. 3A, 5–6; C).

Habitat: Subtidal coarse sands "Amphioxus sand."

Distribution: Europe: Baltic Sea, Kiel Bay, Germany, France (Atlantic and Mediterranean). New records for North America: Cape Cod Bay, Massachusetts;

material received from the Systematics-Ecology Program's Biotic Census; stations: 0612-E1, (1/1); 1412-E4 (23/19); 1812-E2, (209/177); 1910-E1, (2/2); 1930-E2, (1/1); 2008-E5, (4/4). Continental shelf off the coast of North Carolina; station 14 and 15 (3/3).

NOTES ON GEOGRAPHICAL DISTRIBUTION AND HABITAT

The marine Enchytraeidae, in common with Tubificidae and Naididae, are widely distributed and abundant oligochaetes. They live in littoral and subtidal habitats. In Europe many species occur in the Atlantic Ocean, Baltic Sea, the Mediterranean and Black Seas. The Enchytraeidae of other continents have not been studied to the same extent; the fact that species have not been reported from outside European countries certainly does not indicate that their distributions are restricted to Europe.

TABLE I

Enumeration of meiobenthic Enchytraeidae collected from exposed and semi-protected beaches in Massachusetts and North Carolina (Spring 1969). Number of beaches examined is in parentheses

Species	Average numbers of animals per 500 cc of sediment			
	Massachusetts		North Carolina	
	Exposed beaches	Semi-protected beaches	Exposed beaches	Semi-protected beaches
E. capitatus	13 (1)	139 (3)	5 (1)	26 (3)
M. southerni	2 (1)	10 (3)	13 (1)	8 (3)
M. spicula	122 (1)	173 (5)	18 (5)	158 (3)
M. subterranea	8 (1)	464 (5)	9 (6)	26 (3)
M. achaeta	3 (1)	34 (3)	8 (4)	23 (4)
M. preclitellochaeta	170 (1)	215 (4)	9 (1)	83 (1)
Total genera	2	2	2	2
Total species	6	6	6	6
Total individuals/500 cc	436	1035	62	324

Eleven species, described from Europe, widely distributed on this continent, were hitherto unknown from the eastern coast of North America. Two species are apparently limited to Cape Cod Bay, Massachusetts: *Lumbricillus codensis* nov. sp. and *Marionina welchi* nov. sp. The specimens of cosmopolitan species collected by the author in Europe and eastern North America (Massachusetts and North Carolina) are morphologically inseparable.

Both littoral and sublittoral biotopes are inhabited by Enchytraeidae. Four categories of habitats were investigated.

(a) Salt marshes, situated above the upper part of the shore in the supralittoral area where low salinities occur. The dominant species are *Henlea ventriculosa, Cernosvitoviella immota* and *Enchytraeus albidus.*

(b) Gently sloping beaches where decayed seaweeds are scattered in and on the sand or on the gravelly sediment between the high and low tide marks. Four abundant species live in this habitat; *Enchytraeus capitatus*, *Lumbricillus lineatus*, *Marionina spicula* and *Marionina southerni*.

(c) Intertidal sand beaches which possess meiobenthic communities of oligochaetes have a well-sorted sediment and a very small amount of detritus. The species diversity and geographical distribution of these communities are fairly well known for the Western and North-western coasts of Europe (Lasserre, 1971). The density for each species, in any 500 cc sample, collected during the study, is given in the Systematic Section. Examples are given in Table I for beaches in Massachusetts and North Carolina. Maximum densities are found in semi-protected beaches; 1035 individuals per 500 cc in Massachusetts, 324 individuals per 500 cc in North Carolina. The exposed strands have only 436 individuals per 500 cc in Massachusetts and 62 individuals in North Carolina. It seems that Enchytraeidae are not so abundant in North Carolina as in Massachusetts, but it will be necessary to compare the populations over their whole life span. Three species, *Enchytraeus capitatus*, *Marionina preclitellochaeta* and *Marionina achaeta* were found in the upper shore; *Marionina subterranea*, as well as *Marionina preclitellochaeta* and *Marionina achaeta*, were collected from the middle to the lower shore, where low salinities may occur, together with the tubificid *Phallodrilus monospermathecus* (Knöllner). In the case of both the middle and upper shore populations, the maximum densities were found at depths between 5 and 60 cm from the surface. All these observations are in agree with the studies of Jansson (1966, 1968) and Lasserre (1967a, 1970) on beaches in Sweden and France. All species have a broad range of grain size tolerance. The optimum mean grain size between 0.250 mm and 0.900 mm in Massachusetts and between 0.250 mm and 0.500 mm in North Carolina, with a maximum value of 0.20% of fine particles smaller than 0.063 mm. The sorting coefficient, ranging from 1.25 to 2.20 is close to that of Trask's definition of a well-sorted sediment.

(d) Few, but interesting, Enchytraeidae live in subtidal sediments composed mainly of coarse sands. During the Biotic Census of Cape Cod Bay, in 1968, approximately 100 (of a total of 700) subtidal samples were examined for oligochaetes, only 18 contained Enchytraeidae and seven of these samples, consisting of coarse sediment, contained the species *Hemigrania postclitellochaeta*, which is also present in the "Amphioxus sand" (off Cape Hatteras, North Carolina) and widely distributed in coarse subtidal sediments in Europe (Lasserre 1966, 1967a, b). *Lumbricillus codensis* nov. sp. and *Marionina welchi* nov. sp. live in subtidal sediments of Cape Cod Bay composed of 61.8% to 99.9% of sand, at depth between 2 meters and 19 meters. The optimum mean grain sizes are between 0.466 mm and 0.795 mm with a maximum value of 1.86% of fine particles smaller than 0.063 mm. The sorting coefficient ranging from 0.50 to 0.68 is close to that of Trask's definition of a very well-sorted sediment.

Discussion

The abundance of marine Enchytraeidae in North America is indicated by the fact that in spite of the small number of investigations which have been made in the family, six genera, *Henlea* Michaelsen, *Cernosvitoviella* Nielsen and Christensen, *Enchytraeus* Henle, *Lumbricillus* Ørsted, *Marionina* Michaelsen, *Hemigrania* nov. gen. have been shown to be present on this continent. It seems reasonable to predict that future investigations will reveal many other genera and species. The marine enchytraeidae fauna of the Carolinian province is composed of eurythermic species which are very abundant on the north eastern coast of North America and widely distributed in western and north-western Europe.

Field observations and preliminary experiments showed that American representatives of cosmopolitan species of *Marionina* exhibit great ability to withstand wide fluctuations of temperature and salinity, with minimum ranges of more than 25° C and 15‰. The homeostatic capabilities of the two cosmopolitan species *Marionina spicula* and *Marionina achaeta*, collected in the Bay of Arcachon, France, were investigated experimentally (Lasserre 1969, 1970a, b, 1971). It was found that the overall consumption of oxygen in both species was statistically constant over a range of temperature and salinities spanning those normally encountered. There is some evidence to suggest that cosmopolitan marine Enchytraeidae have a world wide distribution reflecting their wide tolerance of physical factors.

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SUMMARY

1. Marine Enchytraeidae from the Eastern coast of North America were examined. Six genera and thirteen species are reported, four genera and eleven species are new to the American continent.

2. One new genus and two new species are described on material received from the Biotic Census of the Systematics-Ecology Program at the Marine Biological Laboratory: *Hemigrania* nov. gen., *Lumbricillus codensis* nov. sp. and *Marionina welchi* nov. sp. Three species are included in the new genus: *Hemigrania postclitellochaeta* (Knöllner, 1934), *Hemigrania monochaeta* (Michaelsen, 1888), *Hemigrania principissae* (Michaelsen, 1907); three species are related to *Marionina welchi* nov. sp.: *Marionina subtilis* (Ude, 1896), *Marionina paucispina* (Eisen, 1904) and *Marionina normani* (Michaelsen, 1907).

3. Marine Enchytraeidae are found in both littoral and sublittoral biotopes. Four categories of habitats are briefly described, (a) salt marshes, (b) the dirftline of seaweed deposit, (c) intertidal sand beaches, with well-sorted sediment and very small amounts of detritus, (d) the subtidal biotopes with very well-sorted sandy sediments.

4. The marine Enchytraeid fauna is composed of eurythermic cold water species, very abundant on the north eastern coast of North America (the Woods Hole region, Massachusetts), and also present under the warmer latitudes of the Beaufort region, North Carolina.

LITERATURE CITED

ALTMAN, L. C., 1931. *Enchytraeus pugetensis,* a new marine Enchytraeidae from Puget Sound. *Trans. Amer. Microscop. Soc.,* **50**: 154–159.

BÜLOW, T., 1955. Oligochaeten aus den Endgebieten der Schlei. *Kiel. Meresforsch.,* **11**: 253–264.

BÜLOW, T., 1957. Systematisch-autökologische Studien an eulitoralen Oligochaeten der Kimbrischen Halbinsel. *Kiel. Meeresforsch.,* **13**: 69–116.

CEKANOVSKAYA, O. V., 1962. Aquatic oligochaetous worms of the fauna of the USSR. *Opredeliteli Faune SSSR Zool. Inst. Acad. Nauk.,* **78**: 3–411.

CERNOSVITOV, L., 1937. System der Enchytraeiden. *Bulletin de l'Association Russe pour les Recherches Scientifiques à Prague* (= Zap. nauchno. issled ob ed. russk. svob. Univ. Prage), **5**: 263–295.

EISEN, G., 1904. Enchytraeidae of the West Coast of North America. *Harriman Alaska Expedition, Alaska Volume 12.* Doubleday, Page and Co., New York, 126 pp.

FREY, H., AND R. LEUCKART, 1847. Beiträge zur Kenntnis der wirbellosen Tiere mit besonderer Berücksichtigung der Fauna des Nord-deutschen Meeres. *Braunschweig,* **4**: 1–170.

HAGEN, G., 1951. Vergleichende ökologische und systematische Untersuchungen der eulitoralen Oligochaetenfauna in Süsswasser-, Braskwasser-und Meeres-gebieten Schleswig-Holsteins. *Dissertation Kiel* (not published) [Cited in Bülow, 1957].

HENLE, F. G. J., 1837. Ueber *Enchytraeus,* eine neue Anneliden-Gattung. *Arch. Anat. Physiol. Med.,* **1837**: 74–90.

HULINGS, N. C., AND J. S. GRAY, 1971. A manual for the study of meiofauna. *Smithsonian Contr. Zool.,* No. **78**, in press.

HUNT, H. R., 1915. Regeneration posteriorly in *Enchytraeus albidus. Amer. Natur.,* **49**: 495–503.

JANSSON, B. O., 1962. Salinity Resistance and Salinity Preference of two oligochaetes *Aktedrilus monospermathecus* Knöllner and *Marionina preclitellochaeta* n. sp. from the interstitial fauna of marine sandy beaches. *Oikos,* **13**: 293–305.

JANSSON, B. O., 1966. Microdistribution of factors and fauna in marine sandy beaches. *Veröeff. Inst. Meeresforsch. Bremerhaven,* **2**: 77–86.

JANSSON, B. O., 1968. Quantitative and experimental studies of the interstitial fauna in four Swedish sandy beaches. *Ophelia,* **5**: 1–71.

KENNEDY, C. R., 1966. A taxonomic revision of the genus *Grania* (Oligochaeta: Enchytraeidae). *J. Zool., London,* **148**: 399–407.

KNÖLLNER, F. H., 1935a. Die Oligochaeten des Küstengrundwassers. *Schr. Naturwiss. Verein Schleswig-Holstein,* **21**: 135–139.

KNÖLLNER, F. H., 1935b. Ökologische und systematische Untersuchungen über litorale und marine Oligochäten der Kieler Bucht. *Zool. Jahrb. Abt. Syst. Oekol. Geogr. Tiere,* **66**: 425–512.

LASSERRE, P., 1964. Note sur quelques oligochètes Enchytraeidae, présents dans les plages du Bassin d'Arcachon. *Procès-verbaux de la Société linnéenne de Bordeaux,* **101**: 87–91.

LASSERRE, P., 1966. Oligochètes marins des côtes de France. I. Bassin d'Arcachon: Systématique. *Cah. Biol. Mar.,* **7**: 295–317.

LASSERRE, P., 1967a. Contribution à l'étude des Oligochètes marins du Bassin d'Arcachon. *Thèse de Biologie, Université de Bordeaux,* 126 pp.

LASSERRE, P., 1967b. Oligochètes marins des côtes de France. II. Roscoff, Penpoull étangs saumâtres de Concarneau: Systématique, Ecologie. *Cah. Biol. Mar.,* **8**: 273–293.

LASSERRE, P., 1969. Relations énergétiques entre le métabolisme respiratoire et la régulation ionique chez une Annélide oligochète euryhaline, *Marionina achaeta* (Hagen). *C. R. Acad. Sci. Paris,* **268**: 1541–1544.

LASSERRE, P., 1970a. Données écophysiologiques sur la répartition des oligochètes marins, méiobenthiques. Incidence des paramètres salinité-température sur le métabolisme respiratoire de deux espèces euryhalines du genre *Marionina* Michaelsen 1889 (Enchytraeidae, Oligochaeta) (*Comm. 3rd. European. Symposium on Marine Biology, 2–7 September 1968*). *Vie Milieu,* in press.

LASSERRE, P., 1970b. Action des variations de salinité sur le métabolisme respiratoire d'Oligochètes euryhalins du genre *Marionina* Michaelsen. *J. Exp. Mar. Biol. Ecol.*, **4**: 150–155.

LASSERRE, P., 1971. Oligochaeta from the marine meiobenthos: Taxonomy and Ecology. In N. C. Hulings, Ed., Proceedings of the First International Conference on Meiofauna. *Smithsonian Contr. Zool.*, No. 76, in press.

MICHAELSEN, W., 1888. Die Oligochaeten von Süd-Georgien nach der Ausbeute der Deutschen Station von 1882–83. *Jahrbuch der Hamburgischen Wissenchaftlichen Anstalten*, **5**: 53–73.

MICHALSEN, W., 1889. Synopsis der Enchytraeiden. *Abhandlungen aus dem Gebiete der Naturwissenschaften hrsg. vom naturwissenschaftlichen verein in Hamburg*, **11**: 1–59.

MICHAELSEN, W., 1900. Oligochaeta. *Das Tierreich, Berlin*, **10**: 1–575.

MICHAELSEN, W., 1907. Oligochaeta. Pages 117–232 in W. Michaelsen and R. Hartmeyer, *Fauna Südwest-Australiens*, **1**. Jena.

MINOR, W. C., 1863. Natural and artificial section on some chaetopod annelids. *Amer. J. Sci.*, **25**: 36.

MOORE, J. P., 1905. Some marine oligochaeta of New England. *Proc. Acad. Natur. Sci. Philadelphia*, **57**: 373–399.

NIELSEN, C. O. AND B. CHRISTENSEN, 1959. The Enchytraeidae, critical revision and taxonomy of European species. *Natura Jutlandica*, **8–9**: 1–160.

NIELSEN, C. O. AND B. CHRISTENSEN, 1963a. The Enchytraeidae, critical revision and taxonomy of European species. *Natura Jutlandica, Supplement 1.*, **10**: 1–23.

NIELSEN, C. O. AND B. CHRISTENSEN, 1963b. The Enchytraeidae, critical revision and taxonomy of European species. *Natura Jutlandica, Supplement 2.*, **10**: 1–20.

PANTIN, C. F. A., 1962. *Notes on Microscopical Techniques for Zoologists.* Cambridge University Press, Cambridge, 77 pp.

SMITH, F., 1895. Notes on species of North American Oligochaeta. *Bull. Ill. State. Lab. Natur. Hist.*, **4**: 285-297.

SOUTHERN, R., 1913. Clare Island Survey, part 48: Oligochaeta. *Proc. Roy. Irish Acad.*, **31**: 1–14.

STEPHENSON, J., 1932. Oligochaeta. I. Microdrili (mainly Enchytraeidae). *Discovery Rep. Cambridge*, **4**: 233–264.

TRASK, P. D., 1932. *Origin and Environment of Source Sediments of Petroleum.* Gulf Publ. House, Houston, 323 pp.

TYNEN, M. AND M. NURMINEN, 1969. A key to the European littoral Enchytraeidae (Oligochaeta). *Ann. Zool. Fennici*, **6**: 150–155.

UDE, H., 1896. Enchytraeiden. *Hamburger Malgalhaensische Sammelreise, Hamburg*, **1**: 1–43.

UDE, H., 1929. Oligochaeta. *Die Tierwelt Deutschlands, Jena*, **15**: 1–132.

UDEKEM, J. D., 1854. Description d'une nouvelle espèce d'*Enchytraeus. Bull. Acad. Roy. Sci. Belgique*, **21**: 864–883.

VERRILL, A. E., 1873. Report upon the invertebrates animals of Vineyard Sound and adjacent waters. *Rep. U. S. Comm. Fish.*, **324**: 622–624.

WELCH, P. S., 1917. The Enchytraeidae of the Woods Hole Region Mass. *Trans. Amer. Microscop. Soc.*, **36**: 119–138.

WELLS, H. W., 1961. The fauna of oyster beds, with special reference to the salinity factor. *Ecol. Monographs*, **31**: 239–266.

Reference: *Biol. Bull.*, **140**: 461–481. (June, 1971)

HISTOCHEMICAL STUDY OF GUT NUTRIENT RESERVES IN RELATION TO REPRODUCTION AND NUTRITION IN THE SEA STARS, *PISASTER OCHRACEUS* AND *PATIRIA MINIATA*[1]

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Studies of the annual reproductive cycle of the sea stars *Pisaster ochraceus* and *Patiria miniata* (Feder, 1956; Farmanfarmaian, Giese, Boolootian, and Bennett, 1958; Greenfield, 1959; Lawrence, 1965; Mauzey, 1966) have indicated that in *Pisaster* the pyloric caeca show a cyclic variation in size which correlates inversely with size changes in the gonads while in *Patiria* the caeca remain relatively constant in size throughout the gonadal cycle. Biochemical changes in gut lipid, protein, and glycogen in rhythm with the reproductive cycle in these sea stars have been reported in Greenfield, Giese, Farmanfarmaian and Boolootian (1958), Giese (1966a and 1966b) and Allen and Giese (1966).

While biochemical studies give quantitative information about the chemical constitution of various body components, limitations in the method generally make it impossible to locate particular substances within particular tissues or cell types within an organ, and highly localized concentrations of a substance may remain unnoticed (Nimitz and Giese, 1964). Histochemical techniques are capable of localizing chemical components into specific cell types and thus are useful complements to biochemical studies. Histochemical studies are in turn dependent on histological and anatomical studies.

The anatomy and histology of the digestive system of *Patiria* have been described by Anderson (1959, 1960), whose work also includes some histochemical observations. The anatomy and histology of the digestive system of *Pisaster* correspond rather closely to descriptions of the digestive system of *Asterias forbesi* in Anderson (1953, 1954). Mauzey (1966) includes both histological and histochemical observations on the pyloric caeca of *Pisaster*.

The purpose of this paper is to report the results of histochemical investigations on the distribution of carbohydrate and lipid substances in the gut of *Pisaster* and *Patiria* in relation to the reproductive cycle and long term starvation. Observations on the histochemistry of mucins and proteinaceous granules are also reported. Similar studies on the gonads and body wall tissues will be reported elsewhere.

MATERIALS AND METHODS

Samples of ten sea stars of each species were collected at roughly monthly intervals for two years. The specimens of *Pisaster* were obtained at Duxbury Reef, northwest of San Francisco in Marin County, and the specimens of *Patiria* from White Gulch on the southwest side of Tomales Bay, Marin County. The animals were maintained in laboratory aquaria at approximately 13° C and processed within forty-eight hours.

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After each animal was weighed, the gonads and pyloric caeca were weighed in order to obtain the gonad and pyloric caecal indices. The indices are ratios of the weight of the gonads or caeca × 100 to the weight of the animal. The indices of ten animals were averaged to obtain each monthly index except in a few instances when poor collecting conditions made it impossible to obtain the full quota of animals.

TABLE I

Histological and histochemical techniques employed

Technique	Purpose	Source
Periodic acid-Schiff (PAS)	Most carbohydrates other than acid mucopolysaccharides	McManus, 1948; Hotchkiss, 1948
Standard sulfation: sulfuric-acetic acid; methylene blue at pH 2.4 (SS)	Carbohydrates other than glycogen	Lewis and Grillo, 1959
Glycogen sulfation: sulfuryl chloride: methylene blue at pH 2.4 (GS)	Glycogen (also stains some other carbohydrates)	Lewis and Grillo, 1959
Lithium-silver nitrate (LiAg)	Glycogen and some mucins	Gomori, 1946; Arzac and Flores, 1949
Alcian blue (AlcBl) and neutral red (NR)	Acid mucopolysaccharides (acidity due to —COOH?)	Steedman, 1950
Azure A metachromasia (AzA)	Acid mucopolysaccharides	Kramer and Windrum, 1954
Methylene blue at pH 2.4 without sulfation (WS)	Sulfated acid mucopolysaccharides	
Sudan III and IV in acetone-alcohol (SOH)	Neutral lipids	Kay and Whitehead, 1941
Oil red O in isopropanol (ORO)	Neutral lipid	Lillie, 1944
Sudan black B in propylene glycol (SBB)	Neutral lipid	Chiffelle and Putt, 1951
0.1% hematoxylin (CH) at pH 3 and Sudan black B (CSBB) on parallel paraffin sections after chromation	Phospholipids	Elftman, 1954
Sakaguchi's method for arginine (SfA)	Protein (arginine-containing)	Baker, 1947
Coupled tetrazonium reaction using H acid (CT)	Protein	Danielli, 1947
Bromphenol blue (HgBB)	Protein	Mazia, Brewer and Alfert, 1953; Bonhag, 1955
Prenant's triple stain; eosin (E), fast green (FG), and Harris's hematoxylin (HH)	Histology	Gabe and Prenant, 1949
Mallory's phosphotungstic acid-hematoxylin	Histology	

Samples of pyloric caeca, intestinal caeca, cardiac stomach floor and pyloric stomach roof taken from two males and two females each month were fixed in Rossman's fluid for general histology and preservation of carbohydrates and in

TABLE II

Histochemical results. The key to the abbreviations for the staining techniques is in Table I. In addition DL stands for diastase-labile and DR for diastase-resistant

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Widely distributed tissues

- Peritoneal cell cytoplasm
  - Fine granules or diffuse staining: FG+; SS+; PAS+; GS+; LiAg+; DL.
  - Irregular yellow granules: NR+; GS bluish green; AzA-β; CSBB+.
- Muscle
  - Fibers: HH+; NR orange; E+; CH+; CSBB+.
  - Fiber sheathing
    - Fine granules: PAS+; GS+; LiAg+; DL.
    - Fine globules: ORO+; SOH+; SBB+.
- Connective tissue fibers: FG+; SS+; PAS+; GS+; AlcBl+; AzA-β.
- Mucous cells
  - Mucus: PAS+; GS+ (often purplish); SS+ (often purplish); AlcBl+; AzA-β; DR.

Cardiac stomach floor

- Typical cell cytoplasm
  - Fine granules or diffuse staining: PAS+; GS+; LiAg+; DL.
  - Fine globules: ORO+; SOH+; SBB+.
- Specialized cell cytoplasm
  - Fine granules or diffuse staining: PAS+; GS+; LiAg+; DL.
  - Fine granules at luminal border: GS+ (purple); WS+; AzA-β; CSBB+; SBB brownish black

Pyloric stomach roof

- Typical cells
  - Fine granules or diffuse staining: PAS+; GS+; LiAg+; DL.

Intestinal caeca

- Typical cells
  - Fine granules or diffuse staining: SS+; PAS+; GS+; DL.
  - Fine globules basally: ORO+; SOH+; SBB+.
  - Yellow granules, 1–1.5 μ, scattered or in a band: PAS+ rarely.

Pyloric caeca

- Storage cell cytoplasm
  - Basal granules of flagella: E+.
  - Storage granules; 1 μ, in rows supranuclearly: FG+; CH+; CT+; SfA+.
  - Fine granules, scattered or in a reticulum or dense reticulum without distinct granules, or diffuse staining: SS+; PAS+; GS+; LiAg+; DL sometimes.
  - Blotchy deposits: SS+; PAS+; GS+; LiAg grayish brown; DR.
  - Globules or diffuse staining: ORO+; SOH+; SBB+.
  - Yellow granules, 0.5–1 μ in a band 4 to 10 μ below border: negative to all stains.
  - Yellow granules, 0.75–2 μ, in a band 12 to 30 μ below border or scattered supranuclearly: negative to all stains.
  - "Other granules": E+; AlcBl+; AzA-β.
- Zymogen cells
  - Granules in conical clusters: E+; CH+; HgBB+; CT+.
- Typical cells of ducts
  - Fine granules or diffuse staining: PAS+; GS+; DL.
  - Fine globules or diffuse staining: ORO+; SOH+.

calcium formol for neutral lipids. Less frequently tissues were fixed in Lillie's neutral formalin for proteins and in 2.5% potassium dichromate in sodium acetate buffer at pH 3.5 for phospholipids. A very few specimens were fixed in Helly's fixative to facilitate study of certain aspects of the histology. Tissues for the study of neutral lipid were embedded in carbowax, other tissues in paraffin, and sectioned at 7 μ.

Table I shows the various staining techniques employed for various purposes and their sources. Control slides for glycogen were incubated in dilute saliva or in 0.01% diastase or amylase in phosphate buffer at pH 6.8 for one to three hours. A few slides of *Pisaster* pyloric caeca were incubated in 0.01% β-galactosidase at pH 7.2, 0.01% β-glucuronidase at pH 7.0, and 0.01% β-glucosidase at pH 5.3 for an hour at 37° C in a series of exploratory tests. A few slides of *Patiria* pyloric caeca were incubated in 0.01% pancreatin at pH 7.0 for one hour.

The samples of tissue from *Pisaster* that had been starved in running sea water tanks at Hopkins Marine Station, Pacific Grove, California, were furnished through the kindness of Dr. A. C. Giese of Stanford University. The specimens of *Patiria* were starved in tanks in the author's laboratory. The water was filtered, aerated and maintained at about 13° C. Sea water and Instant Ocean mix (Aquarium Systems, Inc., Wickliffe, Ohio) were used interchangeably and in combination as convenient.

The starvation for the specimens of *Pisaster* may not have been absolute as some tunicates were discovered living in the aquaria late in the period of starvation (Giese, personal communication). Mauzey (1966) on rare occasions found *Pisaster* feeding on tunicates, though their preferred diet consists of barnacles and molluscs (Feder, 1956; Mauzey, 1966). During starvation both species may have absorbed small amounts of nutrients from natural sea water. Araki (1964) demonstrated the uptake of bovine serum albumin coupled with a diazo salt by *Patiria*, and Ferguson (1967) demonstrated that both *Henricia sanguinolenta* and *Asterias forbesi* absorbed significant amounts of exogenously supplied amino acids and glucose. Even granting the possibilities of nutrients from the sea water or from the stowaway tunicates, it will be apparent that the sea stars under starvation were receiving far less than their normal food intake.

RESULTS

The cardiac stomach of *Pisaster* has five major lobes, each partially subdivided into two portions, one lying on either side of the row of ambulacral ossicles in each arm. The floor of the stomach is thrown into folds radiating from the esophageal region, and these appear in cross section as alternating ridges and gutters.

The wall throughout the digestive tract of *Pisaster* and *Patiria* consists of five layers: a cuboidal epithelium facing the coelom, a layer of muscle fibers, a layer of connective tissue, a nerve layer and the regionally specialized columnar epithelium lining the digestive tract. The basal ends of the columnar cells reach through the nerve layer to attach, often in bundles, to the connective tissue. Amoebocytes with basophilic granules are often seen among the cells of the coelomic lining and in the underlying fibrous layers.

In a study of the cardiac stomach of *Asterias* Anderson (1954) describes "typical cells" with small ovoid nuclei as forming the columnar epithelium of the floor and lower side walls of the gutters. These typical cells are also seen in *Pisaster* in the deeper regions of the gutters. In both *Asterias* (Anderson, 1954) and *Pisaster* specialized cells with elongate nuclei, staining very densely, form the columnar epithelium of the upper side walls of the gutters and intervening ridges, along with mucous cells. As in *Asterias* the line of transition between the typical and specialized cells in *Pisaster* is very abrupt.

At the point near the esophagus where the ridge and gutter system ends and the floor of the stomach becomes a simple, unfolded expanse of tissue in a dissected, relaxed stomach (in the retracted stomach, this region is complexly folded), there is a fairly abrupt transition to a much taller epithelium consisting of very abundant mucous cells intermingled with specialized cells. Present also is another type of secretory cell, reported also by Anderson (1954) in *Asterias*, containing coarse granules which do not stain with phosphotungstic acid hematoxylin. These secretory cells are relatively rare but increase in frequency as one approaches the esophagus proper.

The esophagus in *Pisaster* is most easily distinguished by the fact that its luminal face is regularly ridged, due in part to a folding of the underlying connective tissue and nerve layers and in part to variation in heights of the columnar epithelial cells which are taller in regions overlying the connective tissue folds and shorter between them. The special secretory cells with the refractory granules are relatively abundant in the esophagus and intermingle with mucous cells. However, the most abundant type of cell in the upper region of the esophagus is still the specialized cell with its elongate, densely staining nucleus. Lower in the esophagus the specialized cells are replaced with typical cells containing ovoid granular nuclei. The special secretory cells of the esophagus and adjacent regions of the stomach resemble cells found elsewhere in the external epithelium of the body wall.

This description of the esophagus and cardiac stomach floor in *Pisaster* appears to be in general agreement with Anderson's (1954) descriptions of the corresponding regions of *Asterias*. Anderson, however, describes small granules which stain metachromatically with toluidine blue and are periodic acid-Schiff positive (diastase resistant) at the apical ends of the typical cells (especially those of the lower side walls of the gutters) in *Asterias*. In this present study of *Pisaster* the fine granules staining metachromatically with Azure A appeared to be associated with the specialized cells rather than the typical cells. If present in *Pisaster*, the finely granular cells which Anderson (1954) found in association with the typical cells of the stomach in *Asterias* were not recognized in the present study. Finally, Anderson (1954) does not mention an involvement of the connective tissue layer in the formation of the ridging of the esophagus in *Asterias*.

The columnar epithelium of the roof of the cardiac stomach of *Pisaster* consists of mucous and typical cells, as in *Asterias* (Anderson, 1954).

The pyloric stomach of *Pisaster* is a star-shaped structure with a highly convoluted roof and a simpler floor with five downward folds forming channels radiating from the boundary with the cardiac stomach to the tip of the corresponding lobe. At the tips of the lobes the channels are continuous with short pyloric ducts, each of which soon divides into two and leads into the paired pyloric caeca of each arm. The pyloric duct is interpreted as beginning where the T-shaped sections of the lobe of the pyloric stomach give way to more oval sections.

A discussion of the histology of the pyloric stomach is most easily begun with consideration of the distribution of cell types in the T-shaped cross sections seen near the junction of each lobe with the pyloric duct. In this region the vertical part of the T is the channel which forms the prominent ridge seen externally when looking at the oral surface of the pyloric stomach.

The epithelium lining the channel consists of typical cells with ovoid, granular nuclei interspersed with mucous cells. The nerve layer is very well developed, especially in the depths of the channel, and the columnar cells tallest on the floor of the channel. The nerve layer thins on the side walls and becomes very inconspicuous at the point where the vertical part of the T meets the cross bar. Mucous and typical cells also form the columnar epithelium of the cross bar, but a third type of cell also occurs here. This is a secretory cell characterized by the presence of very fine basophilic granules and a clear vacuole usually in the basal region. Although these cells in many ways resemble the zymogen cells to be described for the caeca, the granules and vacuoles are much smaller than those of the caecal cells.

As one moves towards the center of the pyloric stomach the roof of the cross bar of the T becomes complexly convoluted. The convolutions result in inverted ridges (reaching down into the stomach) and gutters (folding upwards from the stomach). The cells occupying the floors and side walls of the gutters are very crowded and their granular, ovoid nuclei form a broad band, while the cells on the ridges are apparently less crowded and their ovoid nuclei are confined to a band in the middle third of the epithelium. Mucous cells are fairly abundant in all regions of the convoluted roof. The specialized secretory cells, resembling the caecal zymogen cells but with smaller granules and vacuoles, are also found on the crests of the ridges dipping downward into the lumen. The cells forming the columnar epithelium of the floor of the pyloric stomach are the same as those described above for floor regions nearer the pyloric duct.

In the pyloric duct one finds a continuation of the tracts of the same kinds of cells as are present in the adjacent regions of the pyloric stomach. The lower half of the duct is characterized by tall typical and mucous cells with a well developed nerve layer. In the upper half of the duct the nerve layer is inconspicuous and the typical and mucous cells are accompanied by the specialized secretory (zymogen-like) cells.

The pyloric duct is short and soon divides into two ducts which immediately enter the digestive glands. The main duct of each caecum, once within the caecum, is not to be thought of as a discrete rounded channel but simply as a specialized tract of epithelium along the floor and roof of the organ. Branches of the specialized tracts extend into the large pouches, which in turn may be subdivided into smaller ones. The floor tracts consist of mucous cells and crowded typical cells whose ovoid nuclei form a fairly wide band. Corresponding tracts forming the roof consist of mucous and typical cells accompanied by the specialized secretory (zymogen-like) cells. The nerve and connective tissue layers underlying the duct epithelium are especially well developed, but the columnar cells of the ducts are not as tall as those of adjacent pouch regions.

The histology of the esophagus, cardiac stomach, and pyloric caeca with the accompanying Tiedemann's pouches of *Patiria* have been described by Anderson (1959, 1960). The present paper does not intend to repeat but only to supplement information given there, thus attention will be directed to those aspects of the histology which Anderson (1960) treated most briefly.

The wall of Tiedemann's pouch in each caecum falls into two principal regions, one forming the floor and lower side walls of the pouch, and the other forming the upper side walls of the pouch. The lower region is characterized by a nerve layer which increases in thickness towards the floor of the pouch, by an abundance of mucous cells, and by an abundance of typical cells with finely granular, elongate nuclei which form a band in the middle third of the epithelium. The mucous cells are recognized both because of the pockets containing a secretion staining with fast green in Prenant's triple technique and by similar empty pockets. Above the nerve layer and among the basal portions of the typical cells are seen cells, or portions of cells, containing a densely staining, round nucleus about 2 μ in diameter surrounded by granules about 1 μ across and staining with eosin in Prenant's triple technique. The granules are smaller and more irregular looking when stained with haematoxylin in Mallory's phosphotungstic acid-hematoxylin technique. The first impulse of the present author was to interpret these structures as the lower portions of mucous cells in early stages of secretion for the following reasons: (1) known mucus in the distal pockets stains green in Prenant's technique; (2) granules the same size as the granules which stain with eosin in the basal regions of the epithelium are seen in typical mucous pockets distally, colored either beige (transitional ?) or slightly greenish; (3) it is plausible that the staining reaction of the secretion could change as chemical groups are added or unmasked as the secretory material nears the point of extrusion. On the other hand, these structures containing the eosinophilic granules and small round densely staining nuclei also somewhat resemble the cysts which Anderson (1959) describes in the cardiac stomach of *Patiria,* though Anderson makes no mention of finding such cysts in the Tiedemann's pouches in his 1960 paper. A further consideration is that similar structures with granules (though often not so distinct or as large) and round nuclei are seen in the basal part of the epithelium of the floor of the pyloric duct in *Pisaster,* though not in *Pisaster* stomach. The identity of these structures seems unresolvable with the evidence available.

The upper side walls of the Tiedemann's pouches consist chiefly of very crowded typical cells, the nuclei of which form a band extending over the basal two-thirds of the epithelium. At intervals, as pointed out also by Anderson (1960), groups of mucous cells are seen, corresponding in position to the conspicuous lines running from just above the gutter of Tiedemann's pouch up towards the pyloric caecum externally.

There is very little constriction between the cardiac and pyloric stomachs of *Patiria* and for all practical purposes there is no floor to the pyloric stomach. The roof of the pyloric stomach has ten linear upward folds running towards the entrance of the pyloric duct of each caecum. Minor folds directed outward from the wall of the major folds result in a complexly convoluted roof. The openings into Tiedemann's pouches are continuous above with the openings into the pyloric ducts and lie just above the circle of fibers which marks the boundary between the cardiac and pyloric stomachs.

The histology of the pyloric duct (consisting of roof and side walls only, since the duct opens into Tiedemann's pouch along its length) resembles that of the roof of the median duct of the caecum. The columnar epithelium contains typical cells which have elongate, granular nuclei occupying a band across the middle third to basal half of the epithelium, and mucous cells. The epithelium of the roof of the pyloric stomach in regions between and on the lower slopes of the upward folds consists of the same two types of cells, but as one moves upwards, toward and into the minor folds, the typical cells become much more crowded and the nuclear band occupies two-thirds or more of the epithelium. The special secretory cells resembling miniature zymogen cells seen in the roof of the pyloric duct of *Pisaster* are conspiculously absent from the duct of *Patiria,* a fact also noted by Anderson (1960).

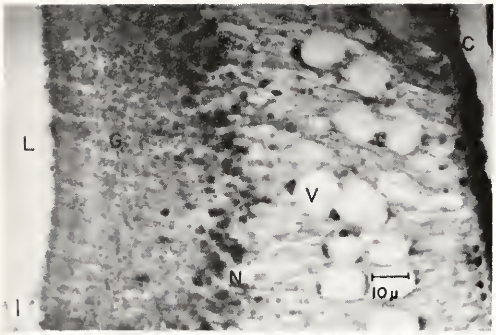
The histology of the pouches of the pyloric caeca of *Pisaster* and *Patiria* resembles that reported for the pouches of other sea stars *(e.g.,* Anderson, 1953, 1966; Chia, 1969). Mucous cells are found in the epithelium of the pouches of the pyloric caeca, but the predominant cell here is a storage cell with a granular, ovoid nucleus containing a prominent nucleolus. The nuclei form a band a little above the middle third of the epithelium. The storage cells contain numerous granules of different types at different times, and little progress was made in the elucidation of the nature and functions of the different types of granules indicated in Table II, with the exception of the so-called storage granules. These large granules (Fig. 1) are about 1 μ in diameter and occur in a clearly defined row in each cell in the supranuclear cytoplasm. The storage granules were present in most specimens of *Pisaster* except those collected in April and May. Mauzey (1966) found also that the granules were abundant in September but absent in April, and related their presence and absence to "seasonal patterns of transfer and storage of energy-rich materials in the hepatic caeca." (page 139) Chia (1969) found that similar granules in *Leptasterias* disappeared from the pyloric caeca during the brooding period. The storage granules are present in *Patiria* but the seasonal pattern of occurrence is not known.

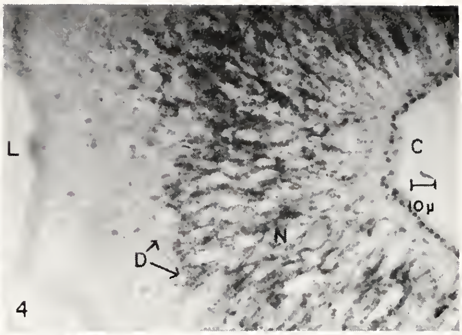
A third type of cell seen in the epithelium of the pockets of the pyloric caeca is the so-called zymogen cell, reported also by Mauzey (1966) and seen in numerous other sea stars (Anderson, 1953, 1960, 1966; Chia, 1969) though not always with the same distribution as in *Pisaster* (Anderson, 1960). The zymogen cells are most easily identified by their cone-shaped clusters or files of 1.2 μ granules. These clusters are occasionally associated with a small clear vacuole, 2 or 3 μ in diameter, but more often are associated with large clear vacuoles, 8 to 12 μ across, which have presumably enlarged from the smaller size. These large vacuoles were seen in about three-fourths of the specimens of *Pisaster,* sometimes only in a few cells, but sometimes they were so numerous as to form a conspicuous band infranuclearly (Fig. 1). At times when the vacuoles were numerous zymogen granules could not be seen even with the staining techniques which usually show them (Prenant's triple, in which the granules stain with eosin and therefore are presumably acidophilic). In the Azure A technique the vacuoles were occasionally continuous with a narrow basophilic channel which reached apically. Then again the channels, still continuous with vacuoles, could appear empty but with traces of basophilic material clinging to the edges. Interpreted as possibly a still later stage in a secretory cycle were instances where the narrow channels reaching apically could be seen in cells in which the vacuoles had apparently collapsed. It is therefore proposed that there is a secretory cycle in which the zymogen cells first form the zymogen granules, and that these, in association with the vacuoles, are transformed just prior to release into soluble, enzymatically active fluid, during which transformation the basophilic groups are unmasked. The significance of the vacuole is problematic. The fact that the vacuoles never stained in any test employed whereas both the zymogen granules and secreted fluid did stain (first as acidophilic then basophilic in the present interpretation) seems to render it unlikely that the vacuoles were serving as a site of accumulation of the active material. Also, if there is a growth from small vacuoles to large ones, this could imply that each vacuole is present for a fair period of time. To have active enzymatic material present inside the cell for any length of time seems contradictory to the idea that zymogen granules are a convenient device to avoid intracellular accumulation of enzymes that might digest the cell itself. The second possibility is that after the cell accumulates a supply of zymogen granules, it then accumulates in the vacuole the substance which later acts on the zymogen granules to convert them into active enzymatic material.

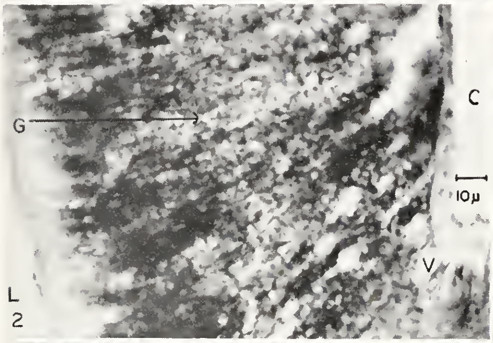
Zymogen cells with granules were seen in about 60% of the specimens of *Pisaster* studied during September, October, November, December, March and July, and in 30% or less of the specimens in most other months. This suggests some correlation with the feeding periodicity observed by Mauzey (1966) who also calls attention to the absence of zymogen granules in specimens of *Pisaster* of low pyloric caecal index. Chia (1969) points out that in *Leptasterias* all evidence of secretory activity in the zymogen cells disappears during the brooding season. Starvation for ten weeks does not alter the secretory activity of the zymogen cells in *Leptasterias* (Chia, 1969), and in the present study zymogen granules were observed in about 60% of the starved *Pisaster* and about 33% of the starved *Patiria*.

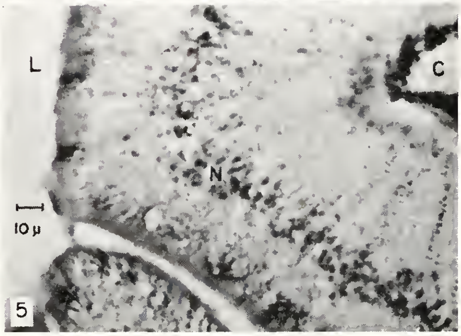
The attribution of an enzymatic function to the so-called zymogen cells and their granules has rested largely on circumstantial evidence (Anderson, 1966). In the present investigation the granules have been shown to stain with the coupled tetrazonium and the mercury bromphenol blue techniques for proteins, and to the extent that this evidence characterizes the granules as proteinaceous it supports a zymogenic function. (It is recognized that the mercury bromphenol blue procedure must be regarded with reservations (Baker, 1958; Kanwar, 1960).

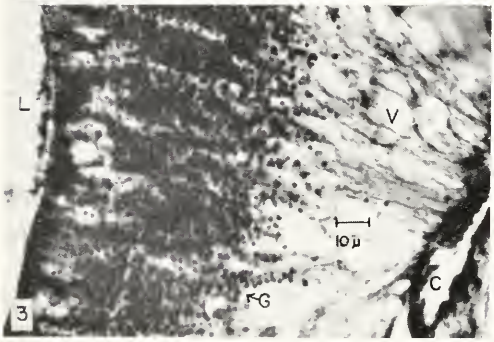
In *Pisaster* the finger-like intestinal caeca are usually three to five in number, 5 to 15 mm long, and about 2 mm in diameter. In *Patiria* each caecum resembles a cluster of grapes, and the individual lobules are about 1 mm in diameter. In the wall of the caeca the muscle layer is especially well developed. Part of the connective tissue layer is "folded" in such a way that it forms the support for the numerous longitudinal ridges which protrude into the lumen. The columnar epithelium lining the caeca consists chiefly of cells which possess elongate, granular nuclei located in the middle third or lower and often numerous yellowish granules from 0.5 to 2 μ across in the supranuclear cytoplasm. These granules may be scattered irregularly or form a distinct band. Mucous cells are also present. Irregular yellowish granules are sometimes seen in the peritoneal cells.











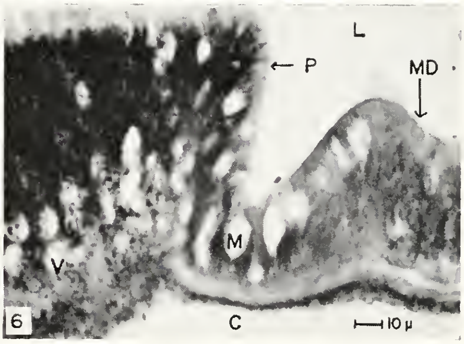


FIGURE 1. Pouch epithelium of the pyloric caeca of *Pisaster*. The nuclei forming a band to the left of the center of the photograph (N) belong to the storage cells, and the granules (G) reaching in files from the nuclear band toward the luminal border (L) are the storage cell granules. The large vacuoles (V) and the nuclei associated with them belong to zymogen cells. The coelom (C) is at the right. This tissue was fixed in Rossman's fluid, embedded in paraffin, sectioned at 7 μ and stained with Prenant's triple technique.

FIGURE 2. Pouch epithelium of the pyloric caeca of *Pisaster*. This tissue was fixed in Rossman's fluid, paraffin-embedded and sectioned at 7 μ, and stained with the periodic acid-Schiff procedure to show the carbohydrate material surrounding the unstained storage granules (G) in the storage cells.

FIGURE 3. Pouch epithelium of the pyloric caeca of *Pisaster*. The storage granules (G) stain deeply with the coupled tetrazonium technique for proteins after fixation in Lillie's neutral formalin, paraffin embedding, and sectioning at 7 μ. Other letters used indicate the same structures as in Figure 1.

From a survey of Table II, summarizing the histochemical staining patterns in the gut of *Patiria* and *Pisaster*, it can be seen that there is evidence for widespread occurrence of a diastase-labile carbohydrate in the tissues of these animals. The carbohydrate material, occurring usually as very fine granules 0.2 μ in diameter, is found in the typical cells of the cardiac stomach floor (and in *Pisaster* also in the specialized cells), in the typical cells of the pyloric stomach roof, basally in the typical cells of the intestinal caeca (though not so consistently here) and in the cells of the duct epithelium of the pyloric caeca. The diastase-labile carbohydrate may well be glycogen, but since current knowledge of the carbohydrates of the invertebrates is still relatively scanty it is perhaps wiser to characterize the carbohydrate that reacts like glycogen as simply glycogen-like. Anderson (1959) also reports a general distribution of glycogen (or a glycogen-like compound) in the stomach of *Patiria;* Anderson (1954) indicates the presence of glycogen in certain regions of the stomach epithelium in *Asterias*.

When very little material reacting positively to stains for carbohydrate is present in the storage cells of the pockets of the pyloric caeca of *Patiria* and *Pisaster,* the carbohydrate here also appears as fine granules labile to diastase, amylase, or saliva. But as the deposits of carbohydrate increase in the storage cells individual granules are no longer recognizable, and the very intensely staining deposits are refractory to diastase, amylase, or saliva to the extent that little or no difference can be seen between slides kept in buffer and slides subjected to one to three hours of enzymatic digestion. This carbohydrate was not labile to β-galactosidase, β-glucuronidase or β-glucosidase in exploratory tests run on *Pisaster* caeca. The intensely staining carbohydrate was absent in sections of *Patiria* caeca after incubation with pancreatin. Anderson (1953) also found that pancreatin removed the diastase-resistant carbohydrate in the caeca of *Asterias* and suggested that the carbohydrate might be bound to protein. Chia (1969) has reported a carbohydrate refractory to diastase in the ground cytoplasm of the storage cells of *Leptasterias.*

The appearance of the carbohydrate material in the storage cells varies in large part due to the presence, absence, or abundance of various kinds of granules and globules refractory to the carbohydrate strains. For instance, at times the carbohydrate may appear simply as fine granules or as a granular reticulum surrounding uncolored storage granules. The reticulum may be so dense that the individual granules composing it are no longer visible (Fig. 2), and there may or may not

FIGURE 4. Pouch epithelium of the pyloric caeca of *Pisaster.* Fine lipid droplets (D) staining with oil red 0 can be seen in the lower two-thirds of the epithelium intermingling with the storage cell nuclei (N). The lumen is at the left, the coelom at the right. The tissue was fixed in calcium-formol; and carbowax sections were cut at 7 μ.

FIGURE 5. Pouch epithelium of the pyloric caeca of *Pisaster.* This tissue was taken from an animal that had been starved for fourteen months, fixed in Rossman's fluid, paraffin-embedded, sectioned at 7 μ, and stained in the periodic acid-Schiff procedure. The nuclei (N) of the storage cells form a prominent band, and there is very little staining for carbohydrate. This figure should be compared to the left half of Figure 6.

FIGURE 6. Pouch (P) and median duct (MD) epithelium of the pyloric caeca of *Pisaster.* This tissue was fixed in Rossman's fluid, paraffin-embedded, sectioned at 7 μ, and stained in the periodic acid-Schiff procedure. Note the intensity of staining in the pouch epithelium; this intensity is typical of the pouch epithelium and is not notably reduced by prolonged diastase- or amylase-incubation. The intensity of staining is much less in the duct epithelium, and the carbohydrate here is diastase- and amylase-labile.

be blotchy deposits of the carbohydrate in the infranuclear region of the cells. At other times the carbohydrate staining of the cells is so uniform as to imply the absence of the refractory storage granules. The amount of carbohydrate may vary from region to region in a single tissue slice, but especially in *Pisaster* it tends to be uniform thoroughout.

This present account of the carbohydrates in the pyloric caeca of *Pisaster* conflicts with Mauzey's (1966) description of carbohydrates in the same species. Mauzey's paper indicates that there are two bands of storage granules supranuclearly in the pocket epithelium and that the more proximal of these bands consists of storage granules which are periodic acid-Schiff positive. Chia (1969) also reports that the storage granules in the storage cells of *Leptasterias* are periodic acid-Schiff positive. The present author characterizes these granules as uncolored by the periodic acid-Schiff technique, though they are embedded in very deeply staining material (Fig. 2). The storage granules were found in the present study to be colored in Sakaguchi's reaction for arginine, the coupled tetrazonium reaction (Fig. 3) and the mercury bromphenol blue reaction, which would suggest that they contain protein. Chia (1969) also reports a protein component in the storage granules of *Leptasterias*.

The more distal band of granules which Mauzey (1966) describes as staining yellow in Mallory's triple technique may be equivalent to the small naturally yellow (*i.e.*, yellow without staining) granules seen in various patterns of distribution in various techniques by the present author.

The present study showed no histochemically detectable variation in the amount of carbohydrate material in the ground cytoplasm of the storage cells of the pyloric caeca, or in any other part of the gut correlating with the reproductive cycle although there is biochemical evidence for such a change (Giese, 1966a and b) which will be discussed in a later section. As mentioned above, Chia (1969) and Mauzey (1966) found that the storage granules, which they characterized as containing carbohydrate, disappeared cyclically, in harmony with the reproductive cycle.

Histochemical results indicate the presence of neutral lipid in the typical and specialized cells of the cardiac stomach floor, typical cells of the pyloric ducts, typical cells of the intestinal caeca, and storage cells of the pyloric caeca (Fig. 4). Anderson (1954) also found lipid in the cardiac stomach epithelium of *Asterias*. In the Tiedemann's pouches of *Patiria* lipid droplets were present in the basal region of the typical cells of the upper side walls, being especially abundant in the cells adjacent to the lines of mucous cells. This is in agreement with Anderson's earlier (1960) observations on the Tiedemann's pouches of *Patiria*. The neutral lipid stains in *Pisaster* and *Patiria* were either taken up by fine globules or appeared to stain diffusely. The deposits were not impressive except in the case of the storage cells of the pyloric caeca. Here discrete droplets of lipid 0.5 to 4 μ in diameter (the larger droplets had probably arisen by the coalescence of smaller ones during processing) occurred almost always in the subnuclear region, and when very abundant, in the supranuclear region as well. Fine lipid droplets were occasionally seen in the peritoneum of the intestinal caeca and stomach.

The distribution of lipids seemed fairly uniform throughout a tissue slice in *Pisaster*, less uniform in *Patiria*. Individual differences in the amount of lipid were observed among specimens in a sample, but no seasonal variation in the amount of lipid was detected histochemically. Chia (1969) found that lipid stores in the pyloric caeca of *Leptasterias* were depleted during brooding, a time during which *Leptasterias* does not feed.

Throughout the gut the connective tissue and the mucins appeared to contain both neutral and weakly acid mucopolysaccharide components, or possibly both neutral and weakly acid residues in a single mucopolysaccharide. Fine lipid globules and granules of glycogen were often associated with muscle fibers of various parts of the gut.

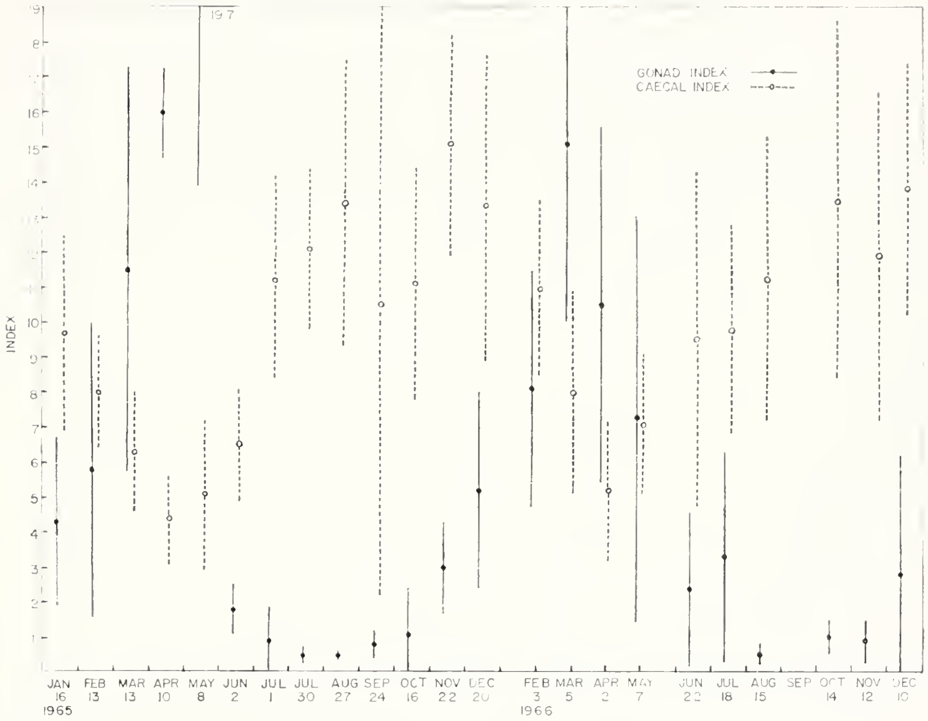


FIGURE 7. Seasonal variation in the gonadal and pyloric caecal indices of *Pisaster ochraceus*. Each mean is indicated by a circle; the standard deviation from each mean is indicated by the vertical line.

Cyclic changes in the pyloric caeca and changes during starvation

Although no seasonal variations in the amount of neutral lipid and carbohydrate stored in the tissues per unit area were detected histochemically, there is evidence that in *Pisaster* the size (and therefore probably also total amount of stored nutrients) of the pyloric caeca varies during the annual reproductive cycle.

The caeca tend to increase in size from July through December and to decrease in size from January to June (Fig. 7). The reciprocal relationship between the size of the gonads and the size of the pyloric caeca suggests that the increase in size of the gonads during gametogenesis occurs at the expense of materials derived from the caeca, as first proposed by Farmanfarmaian, Giese, Boolootian, and Bennett (1958). Biochemical studies (Giese, 1966a and b) to be discussed later do, however, clearly indicate changes in the lipid and carbohydrate contents of the caeca on a per unit dry weight basis.

The monthly indices for *Patiria* (Fig. 8) show no striking reciprocal relationship between the size of the gonads and caeca. The reason for the lack of reciprocal size relationship is not immediately apparent, but possibly it relates to the omnivorous eating habits of *Patiria.* If suitable food is always available there would seem to be no need to accumulate large stores of nutrients in the caeca prior to the onset of gametogenesis. If periodicity of feeding in *Pisaster* is related to space limitations in the arm and the need to make room for gonadal growth by caecal shrinkage (Mauzey, 1966) the relatively constant size of the caeca in *Patiria* might correlate with more constant feeding. Unfortunately Araki's (1964) study of the feeding habits of *Patiria* did not include a study of the presence or absence of periodicity.

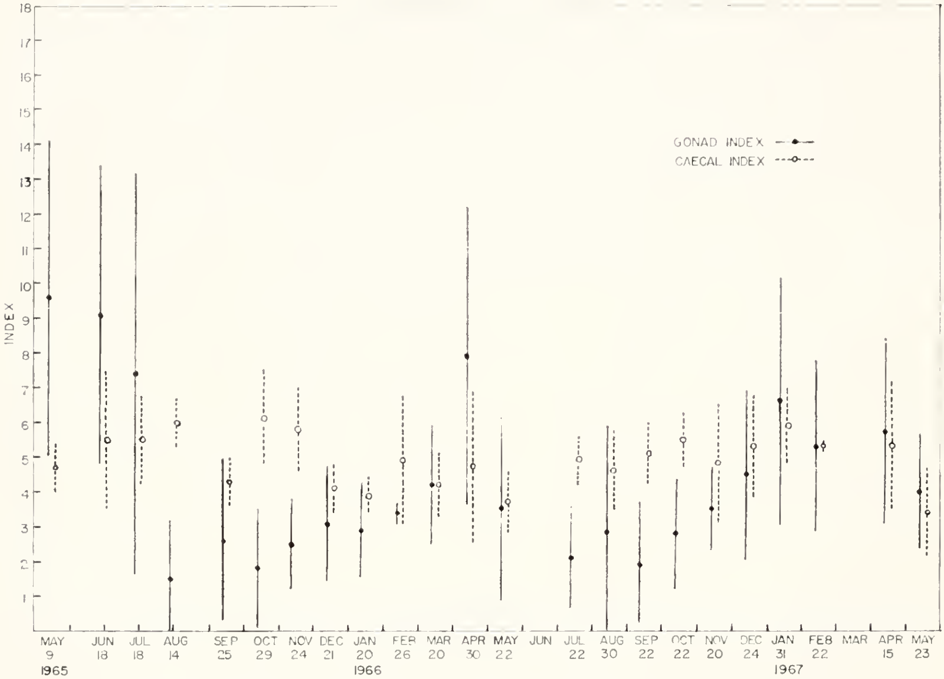


FIGURE 8. Seasonal variation in the gonadal and pyloric caecal indices of *Patiria miniata.* Each mean is indicated by a circle; the standard deviation for each mean is indicated by the vertical line.

Histochemical studies on animals starved for various periods of time support the role of the pyloric caeca as storage organs.

Six specimens of *Pisaster* were starved from December 15, 1964 to February 7, 1966. Of five stomachs processed successfully, four showed almost normal amounts of diastase-labile carbohydrate, while one contained very little carbohydrate. The neutral lipid content in all was much reduced. Pyloric caeca were processed from six animals. Four animals showed a marked reduction in the amount of storage carbohydrate present (Fig. 5; compare with Fig. 6, left half) one showed about normal amounts of carbohydrate, and one was a mosaic of regions of normal and reduced amounts of carbohydrate. Caecal samples for only five animals were processed for neutral lipids, and all five showed a surprising abundance of neutral lipid. However, instead of being confined to discrete droplets this material was diffusely distributed. Thus a starvation period of fourteen months did reduce the carbohydrate content of the caeca in *Pisaster* but did not appear to reduce the lipid content, although it no longer occurred in discrete droplets after processing.

TABLE III

Effects of starvation on the gonadal and pyloric caecal indices of Pisaster ochraceus *and* Patiria miniata *(The means are accompanied by the standard deviations.)*

	Wet weight in grams	Gonad index	Pyloric caecum index
Pisaster ochraceus			
10 animals collected and processed January 16, 1965	244 ± 199	4.3 ± 2.4	9.7 ± 2.8
6 animals starved from December 15, 1964 to February 7, 1966	237 ± 50	0.33 ± 0.25	3.6 ± 0.71
6 animals collected and processed February 3, 1966	227 ± 214	5.4 ± 3.3	5.7 ± 1.5
3 animals starved from December 15, 1964 to July 27, 1966	206 ± 44	0.15 ± 0.02	2.2 ± 0.29
10 animals collected and processed July 18, 1966	411 ± 150	3.29 ± 3.02	9.8 ± 2.58
Patiria miniata			
10 animals collected and processed August 30, 1966	157 ± 46	2.8 ± 3.1	4.6 ± 1.2
11 animals starved from August 30, 1966 to May 13, 1967	109 ± 16	1.8 ± 1.3	1.7 ± 0.04
8 animals collected and processed May 23, 1967	148 ± 49	4.0 ± 1.7	3.4 ± 1.3

Three specimens of *Pisaster* were starved an additional six months till July 27, 1966, making a total starvation period of twenty months. Of stomach samples from all three animals, none showed any carbohydrate and only a very few fine droplets of neutral lipid remained. Of the two pyloric caecal samples successfully processed one showed no carbohydrate and the other only small amounts of carbohydrate. Both animals showed a reduced but still goodly amount of neutral lipid present as small globules or coalesced droplets.

About a dozen *Patiria* were starved from August 30, 1966 to May 13, 1967. Of stomach samples taken from four animals three showed about normal amounts of carbohydrate and one very much subnormal amounts. Of three samples processed for lipids, one had subnormal amounts of lipid and two showed no lipid. Of pyloric caecal samples taken from six animals, five showed little or no carbohydrate and one about normal amounts of carbohydrate. Four showed no neutral lipid, one showed a few droplets, and one almost normal amounts of neutral lipid. The starving *Patiria* had become increasingly inactive, thus reducing their metabolic needs to a minimum.

Insofar as quantity can be estimated histochemically, it is apparent that prolonged starvation reduces the amount of carbohydrate and neutral lipid in the storage cells of the pyloric caeca in both *Pisaster* and *Patiria*. The animals apparently draw on carbohydrate stores before mobilizing the lipid reserves, and it is suggested that carbohydrate stores in the stomach are maintained at the expense of stomach lipid, or possibly also at the expense of carbohydrate or lipid in the pyloric caeca. The endurance of *Pisaster* is impressive until one recalls the presence of the tunicates in the tank. Allen (1964) comments on the survival of *Pisaster* during a nine-month fast during which the animals were kept in running sea water from which microscopic algae were not excluded. The caeca lost weight but the percent lipid content of the caeca remained constant, a finding that corroborates the finding of considerable amounts of lipid in the caeca after starvation in the present study. As Allen points out, *Pisaster*, unlike *Patiria*, does not usually evert its stomach in the absence of macroscopic food but the absence of overt signs of microscopical food intake does not preclude its occurrence. Experiments are necessary in which *Pisaster* is deprived of all possible food sources before its survival limits can be accurately measured.

The specimens of *Patiria* sacrificed after nine months of starvation in the present study appeared to be nearing their tolerance limits; in fact, it was because two animals had apparently succumbed to infection, perhaps due to their weakened condition, that the experiment was terminated at nine months. However, Araki (1964) reports the survival of four specimens of *Patiria* for over eighteen months in running sea water without macroscopic food. The animals surely were getting some food in the form of microorganisms, detritus, and dissolved organic matter; however, he found that the intestinal caeca, pyloric caeca, and gonads all looked emaciated.

Anderson (1953) found that eight weeks of starvation depleted the nutrient stores in *Asterias* while Chia (1969) reports an abundance of storage granules, lipid, and ground substance carbohydrate even after ten weeks of starvation in *Leptasterias*.

The storage function of the pyloric caeca in *Pisaster* and *Patiria* becomes even more evident when one reflects on the reduction in size of the caeca during starvation. The magnitude of this reduction can best be appreciated by referring to Table III. It seems reasonable to assume that the pyloric caecal index of the animals placed under starvation initially would have approximated the pyloric caecal index of the animals taken simultaneously from the field and processed at once. Unfortunately, in the case of *Pisaster* no control animals were processed at once, so that the pyloric caecum index for December 15, 1964 is unknown. The best data available are those for a sample of animals collected a month later at a site 150 miles north of the location from which the animals to be starved were taken. The control sample for the effects of starvation on the pyloric caecal index of *Patiria* was collected at the same place and time as the sample of animals to be starved. Comparing the pyloric caecum indices for *Patiria* collected and processed in August with those starved for nine months, there is a shrinkage from 4.6 ± 1.2 to 1.7 ± 0.04, and for *Pisaster* a shrinkage from 9.7 ± 2.8 in the January "control" animals to 3.6 ± 0.7 after fourteen months of starvation and to 2.2 ±0.3 after twenty months. In all instances the starved animals had significantly smaller caecal indices than field animals collected at the termination of the starvation experiments. (Again the field animals serving as the controls for *Pisaster* came from a population 150 miles north of where the animals under starvation had originally been collected.)

A second criticism to which the data presented are subject is the difference in average size and range of the starved animals and the field animals to which they are being compared. No work has been done with either species on the relationship of body component indices to body size. Giese (1967) found that in the purple sea urchin, *Strongylocentrotus purpuratus*, some body component indices decreased with increasing size of the animal, while others increased.

Despite these criticisms of the data it still seems reasonable to assert that the digestive glands do shrink during starvation as nutrient reserves are withdrawn for use elsewhere. It may then be asked how this shrinkage takes place. Lawrence, Lawrence, and Holland (1965) have found that in the purple sea urchin starvation results in a decrease of the thickness of the epithelial layer lining all major regions of the digestive tract. They have also hypothesized that fluctuations in the thickness of the columnar epithelium of the gut also account for the annual cycle in the gut index. It seems very reasonable to suppose that changes in the height of the columnar epithelium of the pyloric caeca could account for the annual cycle and starvation shrinkage in sea stars as well, but it is futile to attempt to check on this point with tissues available, since one must work with animals of a limited size range to avoid introducing the additional complication that the height of the epithelium might increase with the size of the sea star.

The increase in size of the gonads in *Pisaster* during the time when the pyloric caeca are shrinking (Fig. 7) is interpreted as suggesting that the material withdrawn from the caeca is made available to the gonads. This interpretation agrees with that of Farmanfarmaian, Giese, Boolootian and Bennett (1958).

Prolonged starvation, which would necessitate utilization of nutrient reserves in support of ordinary metabolic needs as well as those of reproduction, would certainly be expected to have an impact on the spring enlargement of the gonads. The data presented in Table III indicate that the gonads fall far short of their ordinary size increases in starving animals.

Discussion

As mentioned in the introduction, it is particularly instructive if biochemical techniques, which are quantitative, and histochemical techniques, which can localize substances within specific tissues or cells, can be brought to bear on the organic constitution of the same animal. It is the purpose of this discussion to examine the histochemical results of this study in relation to earlier biochemical findings.

Giese's (1966b) data for the stomach show that lipid accounts for 15.4% of the dry weight of the stomach in *Pisaster* and 18.4 ± 14.4% in *Patiria*. A glycogen-like carbohydrate accounts for 1.5% of the stomach weight in *Pisaster*, 1.1% in *Patiria*. Again the histochemical results are consistent with these data.

There is a consistent staining for glycogen-like carbohydrate (diastase and amylase labile) in the columnar epithelium of the stomach in both species, and an abundance of fine lipid droplets is seen in most specimens. Part of the biochemically detectable lipid would presumably be structural, and relatively invariable in quantity, and the remainder quantitatively variable lipid reserve. Giese (1966b) sets the value of structural lipid in tissues as roughly 5% of the dry weight as a general average.

Giese's data (1966b) for the pyloric caeca show that in *Pisaster* the lipid content varies from roughly 38% in animals of low caecal index to roughly 18% in animals with high caecal index. The caecal indices tend to be lowest in late spring and highest in the late fall, and the fact that the concentration of lipid in the caeca is highest when the caeca are smallest would seem to suggest that when materials are being withdrawn from the caeca for use by the gonads, some other substance contributing to the bulk of the caeca is withdrawn more extensively than lipid. Allen and Giese (1966) found that the rate of incorporation of labeled precursors into caecal lipid rose during October and November and then decreased till June. Again, if the caecal lipid concentration is lowest when the rate of lipid synthesis is highest, it would appear that some other substance is being accumulated in the caeca at a still greater rate. The data in Giese (1966b) suggest that this substance might be protein. Possibly it is both structural protein associated with an increase in height of the columnar epithelium and protein deposited in the storage granules which do disappear cyclically, as reported by Mauzey (1966) and the present author for *Pisaster*. No cyclic variations in lipid content were noted histochemically, but histochemical results indicate that much of the biochemically detectable lipid occurs as neutral lipid droplets in the columnar epithelial cells of the caeca. That this lipid represents nutrient reserve is demonstrated by its decrease in quantity during prolonged starvation in at least some specimens. The pyloric caeca of *Patiria*, which have no pronounced cyclic variation in size are roughly 22% lipid (dry weight).

Giese's (1966a and b) reviews are not concerned with fractionation of lipid, but Allen and Giese (1966) indicate that ordinarily 70% of the hepatic caecal lipid is neutral lipid, and Greenfield (1959) indicates that the maximum nonsaponifiable lipid fraction observed in his study was 50% of the total lipid. This included considerable amounts of a substance with the spectral characteristics of vitamin D, a carotenoid and a sterol fraction showing seasonal fluctuations and capable of influencing gonad development. Karnovsky, Jeffrey, Thomas, and Deane (1955) found that phosphatides amounted to nearly 40% of the total lipid in the caeca of *Asterias forbesi*, with acetal phosphatides and neutral lipids accounting for the remaining fraction. They found abundant neutral lipid droplets by histochemical techniques, but no phospholipid materials were identified histochemically. Karnovsky attributed the failure of Baker's acid hematein test to demonstrate the phospholipids to the low phosphorus content of the phosphatide fraction. The present author was also unable to demonstrate phospholipid granules of any kind in the caeca of *Pisaster* and *Patiria* perhaps for the same reason, though in this case a differential histochemical test was employed.

Histochemical results show a small amount of diastase-labile glycogen-like material in the pyloric caeca accompanied by large deposits of a storage carbohydrate which is not removed by diastase or amylase. As detectedly biochemically, the content of glycogen-like carbohydrate in *Pisaster* is at maximum 1.7% of the dry weight of the caeca of the male of low caecal index and appears to vary only slightly during the gonadal cycle (Giese, 1966b), while the glycogen-like carbohydrate of the female does show an increase during the cycle from less than 1% in specimens of low caecal index to a maximum of 4.40 ± 3.2% in animals of high caecal index. The content of glycogen-like carbohydrate in the caeca of *Patiria* is also about 1%. The identity of the diastase-refractory carbohydrate is not certain as yet; however, Mr. Arnold Riesen of the author's laboratory has run some preliminary chromatograms of the sugar derived from this carbohydrate. To date, only one sugar appears to be present: glucose. If indeed the carbohydrate proves to be glycogen, it must be assumed that it is bound to some other chemical component in the cells to render it refractory to diastase. As Anderson (1953) has already suggested, this component is possibly protein.

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SUMMARY

1. Histochemical procedures indicate that in *Pisaster ochraceus* and *Patiria miniata* a diastase-labile carbohydrate occurs in small quantities as fine granules in the peritoneum and muscle of the gut, and in the columnar epithelia of the stomach and ducts of the pyloric caecae. A diastase-resistant carbohydrate occurs abundantly in the storage cells of the pyloric caeca, usually as a matrix surrounding large granules of protein. Neutral lipid droplets occur in small quantities in the columnar epithelium of the cardiac stomach and are abundant in the storage cells of the pyloric caeca.

2. In *Pisaster* the pyloric caeca increase in size from June to December and decrease in size during the spring at the time when the gonads are growing. The inverse size relationship suggests the withdrawal of material from the caeca for use by the gonads. The pyloric caeca of *Patiria* seem to remain fairly constant in size during the breeding cycle.

3. In both species prolonged starvation during the breeding period results in shrinkage of the pyloric caeca and reduction of the nutrient reserves to levels insufficient to support normal gonadal development. Starvation most markedly reduces the histochemically detectable carbohydrate in the pyloric caeca. Stomach lipid is reduced in both species, and most of the caecal lipid disappears in *Patiria*. Even after twenty months of starvation *Pisaster* specimens still showed considerable lipid in the caeca.

4. The connective tissue and mucus from most parts of the gut appeared to contain neutral and weakly acid mucopolysaccharide components or a single compound with both neutral and acid residues. Fine granules found at the apices of cells in certain regions of the stomach contain a more acid mucopolysaccharide.

5. The histochemical results correlate well with earlier biochemical data available for the two species.

LITERATURE CITED

Allen, W. V., 1964. Lipogenesis in the sea star, *Pisaster ochraceus*. *Doctoral dissertation, Stanford University*, 173 pp.

Allen, W. V., and A. C. Giese, 1966. An *in vitro* study of lipogenesis in the sea star *Pisaster ochraceus*. *Comp. Biochem. Physiol.*, **17**: 23–38.

Anderson, J. M., 1953. Structure and function in the pyloric caeca of *Asterias forbesi*. *Biol. Bull.*, **105**: 47–61.

Anderson, J. M., 1954. Studies on the cardiac stomach of the starfish *Asterias forbesi*. *Biol. Bull.*, **107**: 157–173.

Anderson, J. M., 1959. Studies on the cardiac stomach of a starfish, *Patiria miniata* (Brandt). *Biol. Bull.*, **117**: 185–201.

Anderson, J. M., 1960. Histological studies on the digestive system of a starfish, *Henricia*, with notes on Tiedemann's pouches in starfishes. *Biol. Bull.*, **119**: 371–398.

Anderson, J. M., 1966. Aspects of nutritional physiology. Pages 329–357 in R. A. Boolootian, Ed., *Physiology of Echinodermata*. New York, Interscience.

Araki, G. S., 1964. On the physiology of feeding and digestion in the sea star, *Patiria miniata*. *Doctoral dissertation, Stanford University*, 182 pp.

Arzac, J. P., and L. G. Flores, 1949. The histochemical demonstration of glycogen by silver complexes. *Stain Tech.*, **24**: 25–31.

Baker, J. R., 1947. The histochemical recognition of certain guanidine derivatives. *Quart. J. Microscop. Sci.*, **88**: 115–121.

Baker, J. R., 1958. Note on the use of bromphenol blue for the histochemical recognition of protein. *Quart. J. Microscop. Sci.*, **99**: 459–460.

Bonhag, P. F., 1955. Histochemical studies of the ovarian nurse tissues and oocytes of the milkweed bug, *Oncopeltus fasciatus* (Dallas). *J. Morphol.*, **96**: 381–439.

Boolootian, R. A., 1966. Reproductive physiology. Pages 591–613 in R. A. Boolootian, Ed., *Physiology of Echinodermata*. New York, Interscience.

Chiffelle, T. L., and F. A. Putt, 1951. Propylene and ethylene glycol as solvents for Sudan IV and Sudan black B. *Stain Tech.*, **26**: 51–56.

Chia, Fu-Shiang, 1969. Histology of the pyloric caeca and its changes during brooding and starvation in a starfish, *Leptasterias hexactis*. *Biol. Bull.*, **136**: 185–192.

Danielli, J. F., 1947. A study of techniques for the cytochemical demonstration of nucleic acids and some components of proteins. *Symp. Soc. Exp. Biol.*, **1**: 101–113.

Elftman, H., 1954. Controlled chromation. *J. Histochem. Cytochem.*, **2**: 1–8.

Farmanfarmaian, A., A. C. Giese, R. A. Boolootian and J. Bennett, 1958. Annual reproductive cycle in four species of west coast starfishes. *J. Exp. Zool.*, **138**: 355–367.

Feder, H., 1956. Natural history studies on the starfish, *Pisaster ochraceus* (Brandt, 1835) in the Monterey Bay area. *Doctoral dissertation, Stanford University*, 294 pp.

Ferguson, J. C., 1967. Utilization of dissolved exogenous nutrients by the starfishes *Asterias forbesi* and *Henricia sanguinolenta*. *Biol. Bull.*, **132**: 161–173.

Gabe, M., and M. Prenant, 1949. Contribution a l'histologie de l'ovogenese chez les Polyplacophores. *Cellule*, **53**: 99–116.

Giese, A. C., 1966a. Lipids in the economy of marine invertebrates. *Phys. Rev.*, **46**: 244–298.

Giese, A. C., 1966b. On the biochemical constitution of some echinoderms, pages 757–796, in R. A. Boolootian, Ed., *Physiology of Echinodermata*. New York, Interscience.

Giese, A. C., 1967. Changes in body component indices and respiration with size in the purple sea urchin *Strongylocentrotus purpuratus*. *Phys. Zool.*, **40**: 194–200.

Gomori, G., 1946. The study of enzymes in tissue sections. *Amer. J. Clin. Pathol.*, **16**: 347–352.

Greenfield, L. J., 1959. Biochemical and environmental factors involved in the reproductive cycle of the sea star *Pisaster ochraceus* (Brandt). *Doctoral dissertation, Stanford University*, 143 pp.

Greenfield, L. J., A. C. Giese, A. Farmanfarmaian and R. A. Boolootian, 1958. Cyclic biochemical changes in several echinoderms. *J. Exp. Zool.*, **139**: 507–524.

Hotchkiss, R. D., 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch. Biochem.*, **16**: 131–141.

Kanwar, K. C., 1960. Note on the specificity of mercuric bromophenol blue for the cytochemical detection of proteins. *Experientia*, **16**: 355.

Karnovsky, M., S. Jeffrey, M. Thompson and H. W. Deane, 1955. A chemical and histochemical study of the lipids of the pyloric caecum of the starfish *Asterias forbesi*. *J. Biophys. Biochem. Cytol.*, **1**: 173–182.

Kay, W. W., and R. Whitehead, 1941. The role of impurities and mixtures of isomers in the staining of fat by commercial Sudans. *J. Pathol. Bacteriol*, **53**: 279–284.

Kramer, H., and G. M. Windrum, 1954. Sulfation techniques in histochemistry with special reference to metachromasia. *J. Histochem. Cytochem.*, **2**: 196–208.

Lawrence, J. M., 1965. Lipid levels in the body fluid, blood and tissues of some marine molluscs and echinoderms in relation to nutritional and reproductive state. *Doctoral dissertation, Stanford University*, 105 pp.

Lawrence, J. M., A. L. Lawrence and N. D. Holland, 1965. Annual cycle in the size of the gut of the purple sea urchin *Strongylocentrotus purpuratus* (Simpson). *Nature*, **205**: 1238–1239.

Lewis, P. R., and T. A. I. Grillo, 1959. Histochemical demonstration of carbohydrates by a convenient sulfation technique. *Histochemie*, **1**: 339–402.

Lillie, R. D., 1944. Various oil soluble dyes as fat stains in the supersaturated isopropanol technique. *Stain Tech.*, **19**: 55–58.

Mauzey, K. P., 1966. Feeding behavior and reproductive cycles in *Pisaster ochraceus*. *Biol. Bull.*, **131**: 127–144.

Mazia, D., P. A. Brewer and M. Alfert, 1953. The cytochemical staining and measurement of protein with mercuric bromphenol blue. *Biol. Bull.*, **104**: 57–67.

McManus, J. F. A., 1948. Histological and histochemical uses of periodic acid. *Stain Tech.*, **23**: 99–108.

Nimitz, Sister M. A., and A. C. Giese, 1964. Histochemical changes correlated with reproductive activity and nutrition in the chiton *Katharina tunicata*. *Quart. J. Microscop. Sci.*, **105**: 481–495.

Steedman, H. E., 1950. Alcian blue 8GS, a new stain for mucin. *Quart. J. Microscop. Sci.*, **91**: 477–479.

Reference: *Biol. Bull.*, **140**: 482–488. (June, 1971)

A NEW LILJEBORGIID AMPHIPOD (CRUSTACEA) FROM KERALA, INDIA

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The single ovigerous female (4.5 mm long) described herein was obtained while examining bottom samples taken during the summer months near the bar mouth of the Kayamkulam lake, a shallow stretch of back water on the west coast of Penninsular India, between latitudes 9°7′ and 9°16′ north and longitudes 76°20′ and 76°28′ east. The main body of the lake runs parallel to the sea from which it is separated by a sandy beach, one to three furlongs wide. The lake communicates with the sea through a narrow bar, except for about four months between January and May. The most noteworthy factor influencing the lake is the periodical changes in salinity, due to tidal influence. The annual variation in salinity may briefly be summarized thus: With the commencement of the dry season early in December, the salinity rises steadily, mainly as a result of tidal influence and partially due to the concentration of salts by evaporation, and continues increasing to about 35‰ in April-May. But with the onset of the southwest monsoon in June, rain water entering the lake through two streams and a commercial canal, the latter bringing in the flood water from the adjoining rivers, raises the water level in the lake by about two feet above the average summer level. Since, by early February the lake becomes cut off from the sea by a bar formed through sand accretion, the newly added flood water dilutes the lake water and thus brings about a marked drop in its salinity. Later, when the bar is cut open to let out the accumulated flood water into the sea, the strong flow flushes out the salinity of the lake completely within three or four days and the lake water thus becomes quite fresh in all its regions, except near the bar mouth where the water is very slightly brackish. This flow of lake water into the sea continues for about three weeks lowering the water level gradually. Simultaneously the tides creep in slowly so that by August-September, the salinity increases to about 15 to 20‰. The northeast monsoon sets in towards the end of September followed by a second flood of lesser intensity during which the salinity again falls for a short period. Since the lake is rather limited in extent, the influence of the tide or that of rain water is immediately felt throughout the lake and it shows an annual variation from 0–35‰.

The floor of the lake is formed of fine sand and silt and is devoid of any natural rock formations. During the summer the bottom supports dense patches of algae in the shallow regions. These weed clusters are favourable habitats for a variety of animals dominated by polychaetes and crustaceans, the amphipoda forming the principal crustacean component. *Listriella similis* sp. nov. is in all probability an inhabitant of such algal communities of the bar mouth where, as suggested earlier, some salinity is detectable even when other regions of the lake are truely fresh water in character. The holotype is deposited at the Marine Biology Laboratory, University of Kerala, Trivandrum-7, India.

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SYSTEMATICS

Suborder: GAMMARIDEA
Family: LILJEBORGIIDAE
Genus: *Listriella* J. L. Barnard
Listriella similis sp. nov.

Female

Body with diffuse pigmentation; cephalon slightly longer than first peraeon segment, anteriorly produced into a small, sharp rostrum, ocular lobes projecting, with irregularly truncate anterior margin, upper angle more pointed. Eyes large, roughly oval and reddish brown. Peraeon segments smooth, deep, the last two longer and deeper than preceding ones. First three segments of pleon subequal in length, depth gradually increasing, ventral margins convex, postero-lateral angle of second segment bluntly produced, distal border convex, with a setule near projection, that of third segment with an upturned dentiform projection followed by a rounded declivity and a setule, the margin above this convex, with a slight notch and a setule at about its middle. Segments 4–6 clearly marked, fourth as long as fifth and sixth combined, sixth distally narrowing and with a small mid-dorsal spine in a shallow depression. Telson twice as long as broad at base, split nearly to $\frac{1}{5}$ of its length, lobes narrow, distally tapering and asymmetrically forked, carrying 2 unequal spines. First coxal plate deeper than corresponding segment, broad and rounded below, second and third shallow, nearly oval, with convex front and lower margins, infero-posterior corners with a small dentiform projection, 4th as broad and deep as first, lower border nearly straight, lateral margins subparallel, hind border with 3 shallow notches, each carrying a setule, upper part excavate. Remaining coxae small and faintly bilobed.

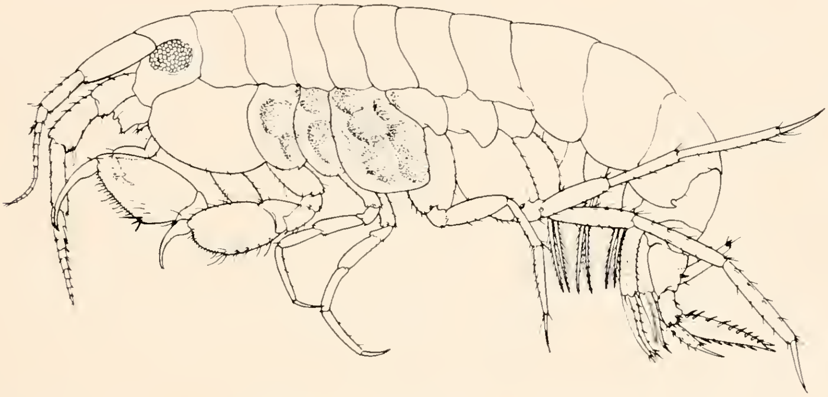

FIGURE 1. *Listriella similis* sp. n., holotype, female, 4.5 mm; Lateral view.

Antennae short; first extending slightly beyond peduncle of second, first segment of peduncle very broad, unarmed and longer than next 2 segments combined, second carrying a few setae on distal and upper margins, third only $\frac{1}{3}$ the length of second, flagellum shorter than peduncle, 10-segmented and moderately setose. Accessory flagellum as long as first 2 flagellar segments combined and 2-segmented. Antenna 2 more setose, peduncular segments spiny along upper margins, second and third segments of peduncle subequal in length and width, gland cone small, fourth and fifth segments subequal in length, flagellum shorter than peduncle, 9-segmented.

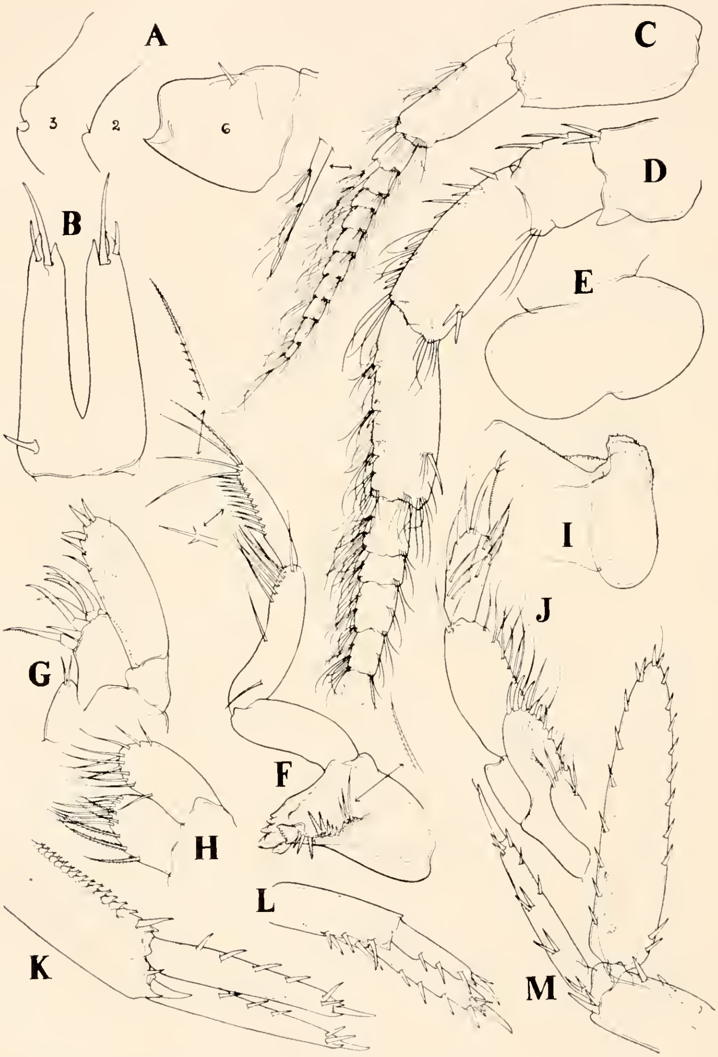

FIGURE 2. *Listriella similis* sp. n. holotype, female, 4.5 mm; A, Pleonal epimera; B, Telson; C, Antenna 1; D, Antenna 2; E, Upper lip; F, Mandible; G, Maxilla 1; H, Maxilla 2; I, Lower lip J. Maxilliped K, Uropod 1; L, Uropod 2; M, Uropod 3.

Upper lip with bilobed distal margin. Incisor process of mandible well chitinized and 5-dentate, lacinia mobilis feeble, cut into several teeth, spine row of 8 short spines, some of them faintly barbed, molar degenerate, represented by a slight prominence carrying 3 pectinate setae. Palp well developed and geniculate between segments 1 and 2, first and third segments subequal in length, former stout and unarmed, second longer and armed with a row of spine-setae on inner distal half, third segment slender, distal $\frac{2}{3}$ of its inner margin carrying a row of short spines, apex armed with 4 long, barbed spine-setae. Inner lobe of 1st maxilla small, conical and armed with 2 short apical setae, outer lobe with 7 well developed pectinate spines, first segment of palp $\frac{1}{3}$ length of second, latter oblong, distally broader and armed with 3 apical spines and a few short spine-setae. Inner lobe of second maxilla slightly wider than outer, but shorter, both apically rounded and armed with setae. Lower lip without inner lobes, outer lobes short and widely separated, distally almost rounded and hairy, with a small inner prominence. Mandibular processes short and rounded. Inner lobe of maxilliped small, armed with a strong spine on inner distal angle, inner border straight. Outer lobe reaching beyond distal margin of first endopod segment, rounded distal and inner borders carrying 7 graduated spines, endopod pediform, first segment $\frac{1}{2}$ length of second, second oblong and armed with a row of long setae, third segment $\frac{1}{3}$ shorter than second, but subequal in length to fourth, pectinate along inner margin and ending in a short pointed nail.

Gnathopods nearly subequal in size; basis of first stout, slightly shorter than propodus, distally narrower, margins armed with long slender setae, ischium almost squarish, as long as merus and armed with a few spine-setae, carpus very small and cup-shaped, propodus oval, naked outer border more than twice the length of inner, latter hirsute, palm oblique, twice the length of inner margin, defined by a group of 3 or 4 spines, palmar border slightly convex and closely armed with short spines interspersed with longer ones. Dactylus long, curved, as long as palm, carrying a few very small setules along inner margin. Basis of second gnathopod nearly of uniform width and more setose, ischium subequal to merus in length, merus rectangular, carpus cup-shaped, much longer than in first gnathopod, shorter inner margin forming a small projecting lobe and armed with a group of setae, propodus oblong, inner margin $\frac{1}{3}$ shorter than outer, both armed with long, spine-setae, in a row on the outer and in fascicles on the inner, palm oblique, convex, defined by a group of spines, nearly as long as inner border and armed as in first gnathopod.

Peraeopods 1 and 2 slender and subsimilar, second slightly larger, basis narrow, subequal in length to next 3 segments combined, sides parallel, ischium small, merus and carpus subequal in length, former slightly broader, propodus very narrow, $\frac{1}{6}$ longer than carpus, dactylus nearly as long as carpus, pointed and slightly falcate. Both appendages sparsely setose. Basis of peraeopods 3 and 4 longer than merus, merus about as long as carpus or propodus, outer margin distally armed with a row of strong spines, inner crenate and distally expanding, ischium very small, merus oblong, margins with a few spines, carpus and propodus slender, former with a row of spines on outer border and apices, latter unarmed on inner margin, outer carrying a row of rather long, slender setae. Dactylus nearly straight, pointed and $\frac{1}{2}$ as long as propodus. Basis of peraeopod 5 as long as propodus and armed as in peraeopod 4, ischium small, merus shorter and both armed with marginal spines. Propodus slender and spiny, dactylus straight, ½ as long as propodus.

Uropods 1 and 2 reaching equally far back; peduncle of 1st as long as the subequal rami, upper margin with a row of tooth-like spines and outer apex carrying a stout spine. Rami apically bidentate and moderately spiny. Uropod 2 shorter than 1st, peduncle as long as longer inner ramus, peduncle and rami spiny. 3rd uropod extending far beyond others, peduncle ½ as long as inner ramus,

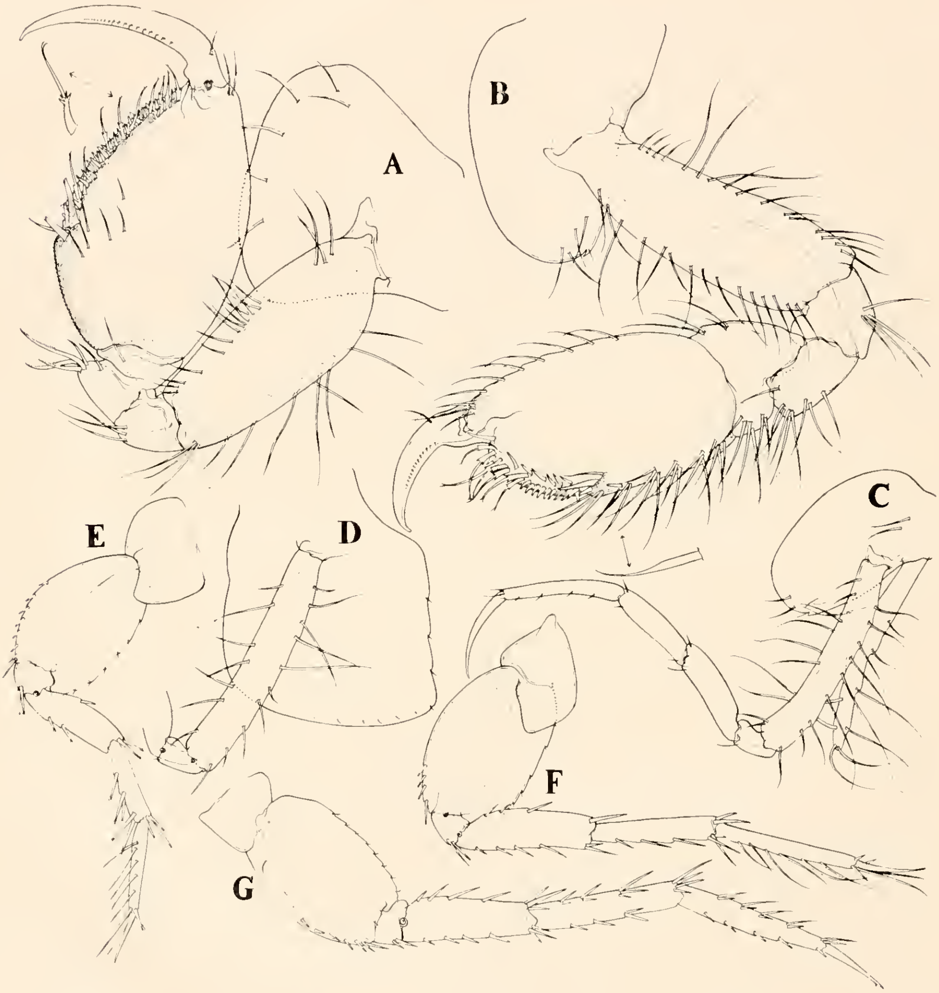

FIGURE 3. *Listriella similis* sp. n., holotype, female, 4.5 mm; A, Gnathopod 1; B, Gnathopod 2; C, Peraeopod 1; D, Peraeopod 2 (articles 2 & 3 with coxa); E, Peraeopod 3; F, Peraeopod 4; G, Peraeopod 5.

latter broad and spiny along both margins. Outer ramus much narrower, slightly more than ½ as long as inner ramus, latter broad and spiny along both margins. Outer ramus much narrower, slightly more than ½ the length of inner and with an apical spiniform segment, less than ½ length of basal segment.

DISCUSSION

In the revised diagnosis provided by J. L. Barnard (1959, page 13) for the family Liljeborgiidae, he mentions among other characters the presence of an elongated first article for the mandibular palp; the geniculation of the mandibular palp between segments 1 and 2 and a telson split right down to its base, as characteristic of the family. Of these, the first two are cited as 'critical points' for the final determination of the members of the family. But subsequently he described a new species *Liljeborgia cota* (J. L. Barnard, 1962, page 83, Fig. 8) in which the palp is not geniculate between segments 1 and 2 and the telson is split only to a third of its length, which is the case in *L. fissicornis* (M. Sars) (see G. O. Sars, 1895, pl. 189) also. Two other liljeborgiids viz. *L. marcinabrio* J. L. Barnard (1969, Fig. 24) and *L. heeia* J. L. Barnard (1970, Fig. 140) also have non-geniculate mandibular palps. Further, the first article of the mandibular palp is not always elongated in all the members of this family (*e.g., L. cota, L. marcinabrio* and *L. heeia*). Thus, it would appear that these three characters cannot go together as diagnositic features of the family.

According to J. L. Barnard (1959), family Liljeborgiidae contains only three genera *viz. Idunella* Sars: *Liljeborgia* Bate and *Listriella* J. L. Barnard. Of these, *Idunella* can be easily distinguished from the other two by the presence of large first gnathopods (larger than second). The distinction between the remaining two genera rests mainly on the structure of the 5th article of the gnathopods since, the biarticulate condition of the outer ramus of uropod 3 mentioned by J. L. Barnard in his key (1959, page 14) is subject to variation as the author himself admits on page 16. To add to this we may also consider the biarticulate nature of the accessory flagellum as a distinctive feature of *Listriella*, whereas it is multisegmented in *Liljeborgia*. Variations in the nature of the accessory flagellum are not unknown among members of the families of suborder Gammaridea (*e.g.*, Isaeidae—including Photidae) and discovery of additional species of both genera may invalidate this character. But, at present this appears to be a very useful difference.

From the females of the hitherto described species of this genus, *Listriella similis* sp. nov. can be distinguished by the following characters: Postero-lateral angle of pleon segment 2 is bluntly produced and the projection is followed by a shallow incision lodging a setule. Pleon segment 3 is very characteristic, the postero-inferior angle is produced into a strong, apically acute hook, followed by a nearly circular incision lodging a setule and further up there is a shallow depression lodging another setule. In this character the present species shows closest resemblance to *L. picta* (Norman) (see Chevreux and Fage, 1925). These two species generally agree in the structure of the mouth parts also. The 6th segment of the first gnathopod in *L. similis* has a nearly straight, prominently spiny palm defined by two strong spines and the segment steadily narrows towards the finger hinge. The only other species which shows a similar palm is *L. diffusa*

J. L. Barnard (1959). Both these agree to some extent in the shape of the 3rd pleon segment also, but they differ in the shape of the 6th segment of the 2nd gnathopod. In this character my specimen shows some affinity to *L. eriopisa* J. L. Barnard (1959), but the latter can be distinguished by the very short outer ramus of uropod 3.

Gratitude is expressed to Dr. N. Krishna Pillai, Reader, Marine Biology Laboratory, University of Kerala, for his advice and guidance throughout this study. The investigation has been partially supported by a Government of India Fellowship, awarded by the University of Kerala. I thank the Government and the University for the assistance.

Through letters, the author has been aided by Dr. J. L. Barnard, Curator, Smithsonian Institution Washington, in certain matters relating to the taxonomy. I thank him most cordially.

Summary and Conclusions

A new gammaridean amphipod, *Listriella similis* is described in detail. This species shows resemblance to *L. picta* (Norman), *L. diffusa* J. L. Barnard and *L. eriopisa* J. L. Barnard, but can be easily distinguished by the shape of the third pleon epimeron and the appearance of the sixth segment of gnathopods 1 and 2.

In the light of the present study, the observations of J. L. Barnard (1959) on the family Liljeborgiidae are critically examined. It is found that the three characters mentioned by him for distinguishing the three existing genera of the family, do not go together. Further, it is suggested that the 2-segmented accessory flagellum of antenna 1 in *Listriella* may serve as an additional character, differentiating it from *Liljeborgia*.

A brief summary of the annual sequence in the salinity fluctuations of the Kayamkulam lake, where from the present species was obtained, is given. It is probable that the association of the animal with the algal communities of the bar mouth region of the lake affords some protection to it in the intertidal.

LITERATURE CITED

Barnard, J. L., 1959. Liljeborgiid amphipods of Southern California Coastal bottoms, with a revision of the family. *Pacific Nat.*, **1**(4): 12–28.

Barnard, J. L., 1962. Benthic marine amphipoda of Southern California: families Tironidae to Gammaridae. *Pacific Nat.*, **3**(2): 73–115.

Barnard, J. L., 1969. A biological survey of Bahía de Los Angeles, Gulf of California, Mexico, IV. Benthic amphipoda (Crustacea). *Trans. San Diego Soc. Natur. Hist.*, **15**(13): 175–228.

Barnard, J. L., 1970. Sublittoral gammaridea (Amphipoda) of the Hawaiian Islands. *Smithsonian Contributions to Zoology*, **34**: 1–286.

Chevreux, E., and L., Fage, 1925. Amphipodes. *Fauna de France*, **9**: 1–488.

Sars, G. O., 1895. Amphipoda. In *An Account of the Crustacea of Norway with Short Descriptions and Figures of all the Species*, Volume 1, 711 pp.

Reference: *Biol. Bull.,* **140**: 489-501. (June, 1971)

LARVAL DEVELOPMENT OF *PAGURUS LONGICARPUS* SAY REARED IN THE LABORATORY. III. BEHAVIORAL RESPONSES TO SALINITY DISCONTINUITIES[1]

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Survival rate, although commonly used as an index of an organism's ability to meet a given environmental challenge (Costlow, Bookhout, and Monroe, 1960, 1962, 1966; Roberts, 1971), is a rather crude estimate of the ability of an organism to adapt to its environment. Behavioral responses associated with the maintenance of an animal in its optimal environment are more suitable measures of adaptability (Shelford, 1915). Orientative behavior reflects, at least in part, the integration of an animal's various adaptive physiological changes in metabolism, reproduction, hormonal balance, *etc.,* to challenges of the external milieu.

Responses of various zooplankters to salinity discontinuities were first studied by Harder (1952a, 1952b, 1954, 1957, 1968). The ability to detect and avoid waters of reduced salinity has now been demonstrated experimentally for several meroplankters as well as holoplankters. A response to salinity discontinuities has been demonstrated in *Mercenaria mercenaria* trochophores (Turner and George, 1955), *Phyllodoce* trochophores (Lyster, 1965), some copepods and the zoeal instars of *Pisidia longicornis* (Lance, 1962), and Zoea I of *Homarus americanus* (Scarratt and Raine, 1967).

In the present study, the response of *Pagurus longicarpus* zoeal instars to salinity discontinuities of different magnitudes was examined. The avoidance behavior was described and quantified. The interaction effect of developmental age and magnitude of the discontinuity on the response was examined. Further an attempt was made to determine if the receptor(s) is localized on certain structures.

MATERIALS AND METHODS

The apparatus used to study responses to salinity discontinuities consisted of two plastic cylinders of 7.9 cm diameter, 46 cm height, marked off into ten 4 cm segments and filled to a depth of 40 cm. The volume of each segment was about 198 ml. A glass tube was cemented at the bottom of each cylinder through which water could be introduced from a separatory funnel. Two centimeters above the bottom of the cylinder, a canula was inserted through a small neoprene stopper, the tip of the canula reaching to the central axis of the cylinder. Externally the canula was fitted with a short plastic tube and pinch clamp to which a large-bore syringe could be attached (Fig. 1).

To establish a discontinuity, about one liter of water of reduced salinity was first poured directly into one column. High salinity water was then slowly introduced at the bottom from the separatory funnel so as to obtain a sharp gradient between the two layers. A preliminary test with dyes indicated that mixing occurred over a distance of about 2–3 mm with little increase in the thickness of the mixed layer through time. The second column (unstratified) served as a control.

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All tests were conducted in a dark room. Light from a 25 watt incandescent bulb was passed through a 3 cm diameter hole in an illuminator located 11.5 cm above the top of each water column, with no lens to concentrate the light beam.

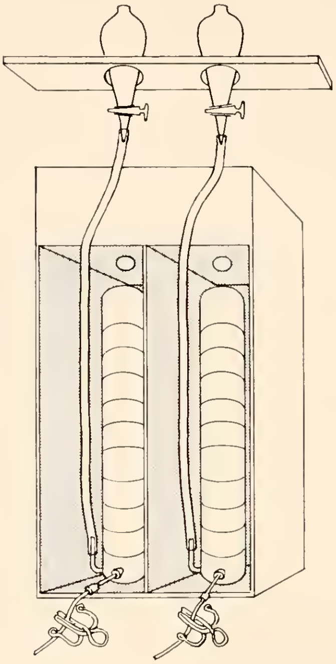

Figure 1. Discontinuity column apparatus, consisting of a plastic cylinder marked off into ten 4 cm segments. A glass tube cemented into the bottom connects to a separatory funnel from which water is introduced. A canula inserted 2 cm above the bottom and connected to a syringe permits injection of the larvae into the bottom stratum.

The cylinders were placed in a compartmented wooden box so that each was isolated from extraneous light and from its twin. No difference in light intensity was detected with a Yentsch photometer between the light sources for each column.

Once the desired salinity discontinuity was established, 50 light-adapted zoeae of the desired stage were drawn into a syringe, the syringe was attached to the canula (previously flushed of air) and the larvae were carefully introduced into the test column. Only on rare occasions were any larvae damaged by this procedure, even the large Zoea IV. The control column was always filled first.

During the first 15 minute period, the larvae were observed and notes taken concerning their movements and behavior. After 15, 30, and 60 minutes, the number of larvae visible in each 4 cm segment was counted and recorded. Some larvae were always invisible, either because they were close to the side of the chamber or on the bottom, but this was shown not to affect the results materially in replicate tests. In addition, larvae moved up and down the column during the counting period, but errors from this source are assumed to be negligible although this was not tested. Because of the depth of field, the height of the column, and distortion resulting from the curvature of the cylinder, a photograph would not have produced a significant improvement in obtaining a quantitative record.

In the main series of tests, the salinity in the lower layer (S_L) was 30, 25, or 20‰. The salinity of the upper layer (S_U) was varied, ranging from $S_U = S_L$ (control) down to a salinity which no larvae would enter, usually 15‰. A specific condition may be abbreviated S_U/S_L. All tests were conducted at 20° C, with no thermal gradient. Controls were alternated between each cylinder.

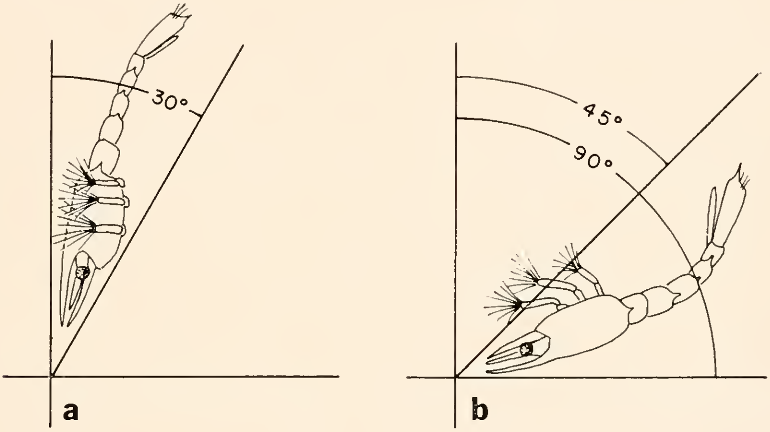

FIGURE 2. Orientation of zoeal body axes (a) when swimming upward or downward, and (b) when sinking passively. See text for further details.

In one experiment, two unstratified columns were established (25/25) with 40 Zoea III in one, 50 in the other, and hourly observations made over a 24 hour period to determine (1) if there was any cyclic pattern to the behavior and (2) if there was a large difference in results between the columns. In a second experiment with two unstratified columns (25/25), 50 Zoea IV were placed in one column, 25 in the other, to determine whether distribution with depth was a function of larval density.

Larvae for all experiments were reared in large mass cultures (larval density: 1 larva per 5–10 ml) of 1000 ml total volume. Larvae were transferred to clean water and fed nauplii of *Artemia* daily. Culture salinity corresponded to S_L for the given experimental series. Culture temperature was 20 ± 2° C.

A brief series of experiments was conducted to determine whether the salinity receptors are located on the antennules and antennae, both of which are known to be involved in chemoreception in adult decapods and other crustaceans. Larvae for these tests were reared at 30‰, 20° C to Zoea III. The antennules (A 1) and antennae (A 2) were then amputated from 12 larvae with fine insect needles. Three additional larvae had the uropods amputated as a control against operative trauma. All amputees were then maintained individually in compartmented plastic boxes at 30‰, 20° C. Survival for the antennectomized group was 50%, for the uropodectomized group 67%. Of the larvae which survived, all successfully molted to Zoea IV. Little or no regeneration was observed in either group of amputees at this stage, but in a series of antennectomies performed on another set of larvae at the same stage of development while developing the operative technique, regeneration of A 1 and A 2 was noted after the molt to the megalopa.

Tests with amputees were performed 24 hours after the operation. The first test was performed to determine whether they could orient in an unstratified column and swim to the surface. Each antennectomized and uropodectomized larva was tested individually for its ability to traverse such a column. Several observations were also made with non-amputees from the same culture. The second test was designed to examine the response of each group of larvae to a stratified column (15/30).

RESULTS

After injection into a control column, zoeae, irrespective of developmental age, oriented with the longitudinal body axis within less than 30° from the vertical, telson uppermost, ventral surface concave downward (Fig. 2a). In this position, they swam directly upward using the natatory setae on the exopods of the maxillipeds. Upon reaching the surface, the larvae lost their precise orientation and swam in circles. Larvae moving downward usually re-oriented and again swam upward.

In stratified columns, zoeae oriented in the same manner, swimming upward to the discontinuity. Discontinuities of only 2.5‰ could be detected and responded to, even though they might ultimately be crossed. After penetrating the upper stratum for 0.5 cm or so, three distinct responses were observed in decreasing frequency in the order described. The most frequent response was a rapid flexion of the abdomen and telson against the ventral surface and re-extension (hereinafter referred to as telson flip response) which caused a disoriented locomotion. If this brought the larva into the lower stratum, it re-oriented and started the cycle over again. If the telson flip response did not return the larva to the lower stratum, and if the upper layer was not so dilute as to cause continued avoidance responses, the larva re-oriented and swam to the surface. The second response, downward swimming, occurred when the upper layer was more dilute, and the zoea remained in the upper stratum after the telson flip response. A 4 cm or more penetration into the upper stratum lasting a considerable period, was followed by the third response, passive sinking. Sinking was distinguishable from active downward swimming by the orientation of the longitudinal body axis which ranged from 45 to 90° from the vertical with the ventral surface concave upward during passive sinking (Fig. 2b).

The percentage of larvae in each 4 cm segment of the column was plotted against depth for each time period. A representative series of data for Zoea I, $S_L = 30‰$, is shown in Figure 3. In the control column, the highest concentration occurred in the uppermost 4 cm segment, a secondary concentration at the bottom, with negligible percentage between. In the 25/30 column, a tertiary concentration was observed in the levels bracketing the discontinuity. In the 20/30 column the primary concentration bracketed the interface with a secondary concentration at the bottom. In the 15/30 column, the primary concentration was located just below the interface with no larvae above the discontinuity. This same general pattern was obtained at every developmental stage in the $S_L = 30$ and 25‰ series, with low S_U causing partial or complete avoidance. The results of the $S_L = 20$‰ series differed in that the primary concentration was always located at the bottom with a secondary concentration at the surface in control columns and at or below the interface in stratified columns.

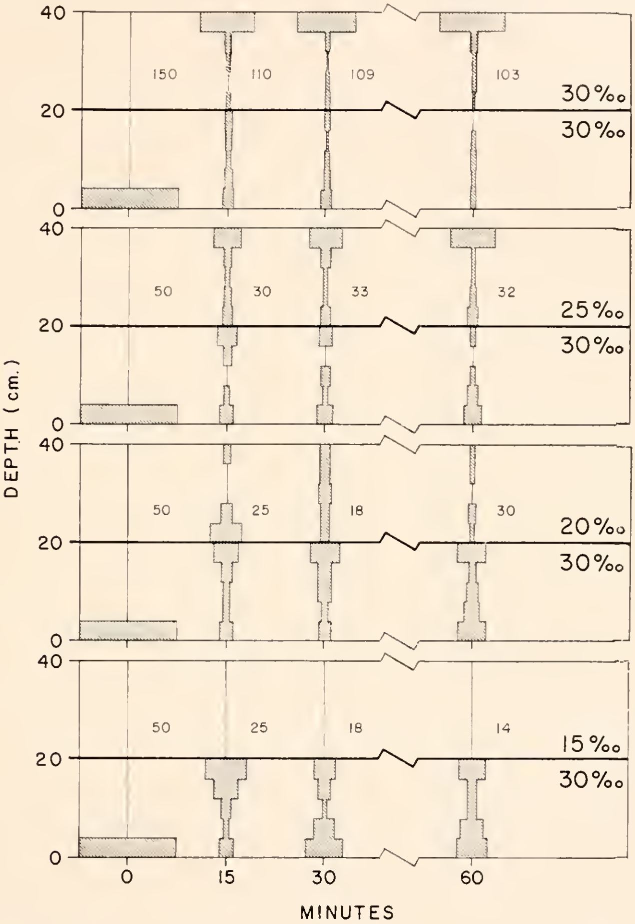

FIGURE 3. Histograms of detailed depth distribution for Zoea. I. The bar in the bottom 4 cm interval for time 0 represents 100%. The number to the right of each individual histogram is the total number of larvae observed in the given time interval and served as the base for percentages.

Disregarding the detailed distribution of larvae, the data were expressed quantitatively in two ways; as an activity index and as an orientation index. The activity index, I_A, is merely the ratio of larvae counted in all 10 levels to the total number of larvae introduced into the column at time t_0 times 100. The orientation index can be expressed as:

$$I_O = \frac{N_U - N_L}{N_U + N_L} \times 100$$

where: N_U = total number of larvae in upper half of column.
where: N_L = total number of larvae in lower half of column.

This index ranges from 100 to −100, with 0 indicating equal distribution between the two layers, and −100 indicating complete avoidance of the upper layer. Lagerspertz and Matilla (1961) used this index in studying the salinity responses of several amphipod and isopod species. The values for both indices in the present study are presented in Table I.

The activity index for control columns was 70–90 for the $S_L = 30‰$ series with a slight increase from Zoea I to IV. In the $S_L = 25‰$ series, I_A for control columns was in the same range but with no apparent increase with developmental age. At $S_L = 20‰$, I_A for the control was lower, 50–70 for Zoea I and II, 60–80 for Zoea III and IV. I_A for each developmental stage decreased as S_U was reduced from $S_U = S_L$ to $S_U = 15‰$ in all three experimental series. The decrease in I_A was more marked in the earlier developmental stages and in the $S_L = 20‰$ series. I_A tended to decrease with time when $S_U = 15‰$, but not at higher S_U's.

The orientation index for control columns at $S_L = 30‰$ and 25‰ was 40 or above except in a few anomalous instances when an unusually high percentage of larvae was present in the lowest 4 cm segment of the column during the early part of the experiment, in which case I_O was *ca.* 10. At $S_L = 20‰$, I_O for the control column was usually much lower, in the 10 to −40 range. Regardless of S_L, I_O decreased with decreasing S_U; in most cases $I_O < -60$ at $S_U = 15‰$. In most tests I_O tended to increase with time whenever the initial value was greater than 0 and to decrease whenever the initial value was less than 0. This trend was apparent regardless of the ΔS $(= S_L - S_U)$.

The most obvious question is whether avoidance of a water mass is a function of an absolute salinity or whether it is a function of ΔS. To examine the possibilities, I_O values for 60 min were plotted on a graph of ΔS *versus* S_L for each zoeal stage (Fig. 4a–d).

If avoidance is based on an absolute salinity, then a line separating the regions of avoidance and nonavoidance would be expressed by $\Delta S = S_L - a$ where $a = S_L$ where $\Delta S = 0$, whereas if avoidance is based on the salinity difference exclusively, such a line would be expressed by $\Delta S = b$ for all S_L. If avoidance is based on one factor over part of the range of S_L and the other over the rest of the range, a family of curves between these two models would be generated.

The avoidance response of Zoea I is based on an absolute salinity of about 20‰ or slightly less when $S_L < 25‰$, and $\Delta S = 8‰$ when $S_L > 25‰$. Similarly, for Zoea II, the avoidance response is a function of an absolute salinity of about 22‰ when $S_L < 25$‰ and $\Delta S = 5$‰ when $S_L > 25$‰. The tendency for the I_0 value for $S_L = 30$‰ to be less than that for $S_L = 25$‰ at each ΔS for these two stages cannot be explained.

For Zoea III and Zoea IV the avoidance response is a function of an absolute salinity of about 20‰. Actually, the slope of the line is steeper than predicted by the first model, but this may result in part from the imprecision of I_0 and the fact that the experiments on which the results are based were carried out over a five month period.

TABLE I

Activity and orientation indices for each developmental stage. The activity index in italic type, the orientation index in roman type

S_U	Time (min)	S_L											
		30				25				20			
		Developmental stage											
		I	II	III	IV	I	II	III	IV	I	II	III	IV
30	15	27 *73*	16 *72*	21 *77*	39 *85*								
	30	42 *73*	13 *72*	18 *70*	41 *92*								
	60	59 *69*	52 *82*	37 *74*	52 *89*								
25	15	7 *60*	−25 *64*	33 *78*	25 *80*	46 *89*	21 *81*	47 *88*	83 *79*				
	30	21 *66*	−15 *66*	54 *70*	19 *84*	60 *95*	31 *87*	49 *92*	72 *86*				
	60	38 *64*	−10 *58*	43 *70*	19 *84*	62 *88*	53 *83*	38 *89*	64 *85*				
20	15	−12 *50*	−86 *84*	48 *62*	−11 *90*	69 *90*	−25 *77*	0 *88*	10 *90*	−11 *60*	−62 *52*	−40 *80*	−37 *73*
	30	−33 *36*	−76 *50*	50 *64*	6 *80*	66 *94*	−19 *76*	−25 *90*	10 *88*	−11 *63*	−66 *59*	−37 *76*	−38 *74*
	60	−63 *60*	−100 *56*	63 *64*	9 *87*	68 *88*	3 *76*	5 *80*	8 *94*	8 *61*	−30 *66*	−38 *77*	−19 *64*
17.5	15									−94 *34*	−60 *30*	−82 *66*	−77 *52*
	30									−95 *37*	−60 *40*	−62 *74*	−63 *32*
	60									−85 *38*	−60 *50*	−55 *62*	−33 *42*
15	15	−100 *50*	−57 *56*	−47 *76*	−31 *70*	−6 *72*	−90 *76*	−47 *75*	−9 *70*		−87 *30*	−93 *54*	−100 *46*
	30	−100 *36*	−72 *50*	−81 *42*	−43 *42*	−11 *72*	−90 *78*	−84 *60*	−37 *76*		−100 *36*	−89 *36*	−79 *38*
	60	−100 *28*	−100 *30*	−86 *28*	−70 *54*	−8 *74*	−100 *60*	−100 *58*	−54 *78*		−100 *28*	−100 *32*	−100 *36*
12.5	15					−65 *34*			−44 *78*				
	30					−63 *32*			−67 *64*				
	60					−76 *34*			−77 *68*				

In the single long-term experiment lasting 25 hours, I_A in two unstratified columns was 80–98 at each observation. For the first 7 hours in one column and the first 2 hours in the other, I_0 was 40–50, increasing to 60–90 for the remainder of the experiment. The shift in I_0 in both cases resulted from a reduction in the percentage of larvae in the bottom-most level. Perhaps these larvae were slightly damaged during the injection into the column, resulting in a negative light response which was slowly reversed with time. In any case, no periodic variation was noted in either I_A or I_0. I_0 did not return to the low initial value of 40 after 24 hours, but remained at the high value of 80 reached some time after the experiment was started.

The effect of larval density was briefly examined in a single experiment. At a density of 1 larva per 40 ml, the density used in the main series of tests, $I_0 = 40$, $I_A = 80$ to 90. When the larval density was reduced by half to 1 larva per 80 ml, $I_0 = 50$ to 60, $I_A = 70$ to 80. A χ^2-test on the data for each 4-cm segment showed no significant difference in the distribution on the two columns, but, as I_0 reveals, there was a slight tendency for a greater proportion of the larvae to enter the upper stratum at the lower overall larval density. I doubt that the use of lower larval densities would have changed the results of the main series just described, however.

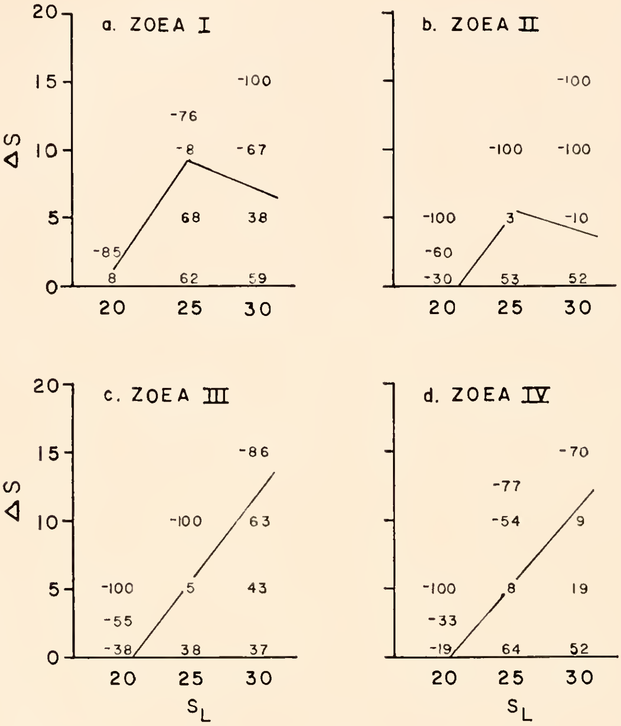

FIGURE 4. The orientation index (I_0) values measured after 60 min are plotted on a matrix of salinity differences across the discontinuity (ΔS) *versus* the salinity of the lower layer (S_L) for each zoeal instar. The solid line in each case approximates the $I_0 = 0$ line. Values of $I_0 < 0$ represent avoidance of the upper water layer, values of $I_0 > 0$ represent nonavoidance.

The results described above were derived from larvae of several females with different prehistories, especially with regard to season. There is no *a priori* reason why larvae from different females, different seasons and different geographical locations should behave in exactly the same manner in response to any given stimulus. However, no attempt has been made to test the effect of these variables. Agreement of results from replicated controls suggests that these variables are unimportant, with the possible exception of geographical location.

In experiments with antennectomized and uropodectomized Zoea III's, the ability of amputees to traverse an unstratified column was tested first. Antennectomized and uropodectomized larvae, after introduction into the column, moved slowly upward. Orientation of the longitudinal body axis varied erratically from 0 to 90° from the vertical. It took 2 to 3 minutes for each larva to reach the surface. Non-amputees from the same culture traversed the column in about 1 minute, with very precise orientation of the longitudinal body axis as described above (Fig. 2a).

In a stratified column (15/30), the behavior of all three groups, non-amputees, antennectomized, and uropodectomized larvae, was the same as that of control larvae in the main series of experiments. Upon encountering the discontinuity, each larva responded with telson flips, downward swimming, and sinking, which resulted in complete avoidance of the upper stratum. Each larva repeated these responses continuously over a 5 minute observation period after which it was removed from the column and another larva introduced.

DISCUSSION

Despite differences in apparatus and procedure, several authors have observed a definite behavioral response to salinity discontinuities and have been able to identify a critical salinity difference causing avoidance. Harder (1968) listed species from seven phyla which have been shown to respond: Protozoa, Ctenophora, Chaetognatha, Annelida (Polychaeta), Mollusca, Arthropoda (Crustacea), and Chordata (Larvacea and Teleostei). To Harder's list may be added *Mercenaria mercenaria* trochophores (Turner and George, 1955), *Phyllodoce* larvae (Lyster, 1965), several copepod species and *Pisidia longicornis* Zoea I and II (Lance, 1962) and *Homarus americanus* Zoea I (Scarratt and Raine, 1967).

Detailed comparisons of responses will only be made among the various decapod crustaceans which have been studied to date. At each developmental stage, the salinity which causes avoidance by *P. longicarpus* is different. The most sensitive stage is Zoea II. Lance (1962) did not observe any difference in the response of the zoeal instars of *Pisidia longicornis*. Scarratt and Raine (1967) made no attempt to assess the effect of developmental age on the avoidance response.

Despite differences in response of each developmental stage, the avoidance salinity was in every case higher than the minimum survival salinity, indeed even the minimum optimal salinity. *P. longicarpus* larvae at every instar partially avoided 20‰ ($I_0 \simeq 0$) and completely avoided lower salinities ($I_0 < 0$). Complete development occurred as readily at 18.0‰ as at 30.5‰ and was still possible at 15.5‰ though with a marked reduction in survival rate. Only at 13.0‰ was survival and development completely halted (Roberts, 1971). *Pisidia longicornis* exhibited more than 50% mortality in 20 hours at salinities below 14.4‰ with 100% mortality at about 12.6‰ for both zoeal instars (Lance, 1964). Both zoeal instars avoided 18‰ almost completely and 14.4‰ completely (Lance, 1962). It should be noted, however, that the mortality rates in the present study and in

Lance's work are not exactly comparable (see Roberts, 1971). Scarratt and Raine (1967) found that *Homarus americanus* Zoea I avoided 21.4‰ although they can survive at least in water of 20.7‰ and perhaps even in water of only 13.5‰ salinity.

Lance (1962) attempted to assess the effect of ΔS by setting up multilayered columns (each layer 5 cm thick) with $\Delta S = 3.6$ or 1.8‰ between each layer. Both zoeal instars concentrated in strata with a salinity between 29 and 32.5‰ with a few entering water of only 25‰ salinity when $\Delta S = 1.8$‰, and only 21.5‰ when $\Delta S = 3.6$‰. Larvae entering water layers of 21.5 and 25‰ salinity were, in my view, merely "sampling" these water masses and were not "permanent residents." Failure to enter water of 18‰ resulted from the larvae having responded already to water of a slightly higher salinity.

In the present study, a different approach was employed; S_L was progressively reduced to determine changes in the salinity which cause avoidance. In this way it was shown that ΔS and absolute salinity both are important in the response of the larvae. Apparently at later developmental stages, ΔS becomes less important. In any case, one would predict that larvae in a multilayered column would concentrate at a level with salinity between 20 and 25‰ with little penetration to levels with salinity between 17.5 and 20‰. This conclusion is derived from the fact that the primary concentration of larvae in experiments with $S_L =$ 20‰ was located at the bottom with a very small percentage near the interface. This prediction agrees with the results of Lance (1962) already noted, although not tested here.

Harder (1952b) noted that zooplankters, mostly copepods, aggregate at the discontinuity between water masses of differing salinity or temperature. In subsequent experiments (1954, 1957), he detected a response by copepods to salinity differences as small as 0.20‰. Even when the discontinuity was produced by disrupting the ionic ratios by the addition of NaCl, copepods responded as in previous tests. When non-electrolytes such as sugar and glycerol were added to the water in varying amounts to produce concentration discontinuities, the animals again responded by aggregating at the discontinuity. He hypothesized from these results that the effective stimulus for the response was density or viscosity. To test this, he set up a stratified column with $\Delta S = 14.3$‰, a sugar difference of 25‰, but no density difference. In this case, no aggregation occurred at the discontinuity. Since the viscosity of the two strata differed, Harder concluded that density provided the stimulus. There are two criticisms of this conclusion, however. First, he has not shown that the density of the stratum avoided was the same for both thermal and saline discontinuities. The response to a thermal discontinuity could be simple orthokinesis (see for example Welsh, 1932), while the response to a saline discontinuity could be to some non-density property. This leads to the second criticism. Harder's results clearly show that the salinity response of copepods is not based on detection of ionic concentration. However, sucrose addition affects all colligative properties, not just density and viscosity. Osmotic pressure could well have been the effective stimulus in producing the response. Further investigation is necessary to clarify this point.

The amphipod, *Gammarus*, and the isopod, *Asellus*, can discriminate between water of 0 and 6‰ (Lagerspetz and Mattila, 1961). Using antennectomized animals, they showed that the sensory receptors are localized on A 1 and A 2 of *Gammarus*, and on one or both of these structures of *Asellus*. In *Pagurus* larvae, however, there is no evidence of localization of receptors on A 1 and/or A 2. Chemoreceptors have been found on the dactyls of the pereiopods and on the maxillae of adult decapods, although these are not necessarily sensitive to salt solutions (Hodgson, 1955). No further efforts were made to isolate potential receptor sites with *P. longicarpus* larvae because of difficulty in successfully amputating suspect structures.

Larvae of *P. longicarpus* are capable of detecting a ΔS of only 2.5‰ and probably less. There are two types of data to support this contention: direct observation of individual larvae and differences in I_0. Larvae passing through any discontinuity used in the experiments exhibited the flip response irrespective of whether or not they ultimately avoided the upper stratum. Further, I_0 decreased stepwise with each 2.5 or 5‰ decrease in S_U regardless of S_L. This latter result is also apparent in the data for *Pisidia longicornis* (Lance, 1962).

The flip response is a clearcut avoidance response or klinokinesis. The sinking response is a cessation of all swimming activity. The downward swimming response, which can at times be very rapid and is well oriented with the longitudinal body axis as in upwards swimming, is not clearly understood. Ewald (1912) concluded that nauplii of *Balanus perforatus* become photonegative when placed in water of reduced salinity. Lyon (1906) had previously concluded the same thing for *Palaemonetes* larvae. In both cases the results can be explained in another way since the experiments were not adequately controlled. In the present experiments, downward swimming could be explained as a reversal of the photo-response, or by the reversal of the georesponse. No attempt has yet been made to discriminate between these possibilities. The results obtained from the $S_L = 20‰$ series suggest that one or both of these responses have been reversed.

Regardless of which response was reversed, larvae tested in columns in which complete avoidance of the upper stratum was noted also had a low I_A. The uncounted larvae could be seen oriented as if swimming but not moving up off the bottom (hence not counted). This suggests a continued active avoidance even though the larvae were in a suitable salinity. Any larva returning to a photopositive (or geonegative) response, would swim upward to the interface and again experience a reversal of the photo-(geo)-response.

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SUMMARY

1. Each zoeal instar of *P. longicarpus* was exposed to salinity discontinuities of varying magnitudes. Three distinct behavioral responses were observed: telson flips regardless of the magnitude of the discontinuity; downward swimming when the magnitude of the discontinuity was moderate; passive sinking when the magnitude of the discontinuity was extreme.

2. All zoeal instars avoided water with a salinity of *ca.* 20‰ or less, well within the range of salinity permitting complete development to the juvenile.

3. Avoidance of low salinity layers in stratified columns by Zoea I and II appeared to be a function of an absolute salinity ($S \simeq 20-22$‰) when the salinity of the lower layer (S_L) was less than 25‰ and a function of salinity difference (ΔS) when S_L was greater than 25‰ ($\Delta S \simeq 8$‰ for Zoea I; $\Delta S \simeq 5$‰ for Zoea II). Avoidance of low salinity layers by Zoea III and IV appeared to be a function of absolute salinity ($S \simeq 20$‰) regardless of S_L over the range of S_L tested.

4. Evidence is presented that the antennules and antennae may not be involved in detection of salinity discontinuities.

LITERATURE CITED

COSTLOW, J. D., JR., C. G. BOUKHOUT AND R. MONROE, 1960. The effect of salinity and temperature on larval development of *Sesarma cinereum* (Bosc) reared in the laboratory. *Biol. Bull.*, **118**: 183–202.

COSTLOW, J. D., JR., C. G. BOOKHOUT AND R. MONROE, 1962. Salinity-temperature effects on the larval development of the crab, *Panopeus herbstii* Milne-Edwards, reared in the laboratory. *Physiol. Zool.*, **35**: 79–93.

COSTLOW, J. D., JR., C. G. BOOKHOUT AND R. MONROE, 1966. Studies on the larval development of the crab, *Rhithropanopeus harrisii* (Gould). I. The effect of salinity and temperature on larval development. *Physiol. Zool.*, **39**: 81–100.

EWALD, E. F., 1912. On artificial modification of light reactions and the influence of electrolytes on phototaxis. *J. Exp. Biol.*, **13**: 591–612.

HARDER, W., 1952a. Einige Geräte zur Herstellung von Wasserschichten verschiedener Dichte. *Kurze Mitt. Fischbiol. Abt. Max-Planck-Inst. Meeresbiol. Wilhelms*, **1**: 21–27.

HARDER, W., 1952b. Uber das Verhalten von Zooplankton in geschichtetem Wasser. *Kurze Mitt. Fischbiol. Abt. Max-Planck-Inst. Meeresbiol. Wilhelms*, **1**: 28–43.

HARDER, W., 1954. Weitere experimentelle Untersuchungen über das Verhalten von Copepoden gegenüber Sprungschichten. *Kurze Mitt. Fischbiol. Abt. Max-Planck-Inst. Meeresbiol. Wilhelms*, **4**: 1–47.

HARDER, W., 1957. Verhalten von Organismen gegenüber Sprungschichten. *Année Biol.*, **33**: 227–232.

HARDER, W., 1968. Reaction of plankton organisms to water stratification. *Limnol. Oceanogr.*, **13**: 156–168.

HODGSON, E. S., 1955. Problems in invertebrate chemoreception. *Quart. Rev. Biol.*, **30**: 331–347.

LAGERSPERTZ, K., AND M. MATTILA, 1961. Salinity reactions of some fresh- and brackish-water crustaceans. *Biol. Bull.*, **120**: 44–53.

LANCE, J., 1962. Effects of water of reduced salinity on the vertical migration of zooplankton. *J. Mar. Biol. Ass. U. K.*, **42**: 131–154.

LANCE, J., 1964. The salinity tolerances of some estuarine planktonic crustaceans. *Biol. Bull.*, **127**: 108–118.

LYON, E. P., 1906. Note on the heliotropism of *Palaemonetes* larvae. *Biol. Bull.*, **12**: 23–25.

LYSTER, I. H. J., 1965. The salinity tolerance of polychaete larvae. *J. Anim. Ecol.,* **34**: 517–527.

ROBERTS, M. H., JR., 1971. Larval development of *Pagurus longicarpus* Say reared in the laboratory. II. Effects of reduced salinity on larval development. *Biol. Bull.,* **140**: 104–116.

SCARRATT, D. J., AND G. E. RAINE, 1967. Avoidance of low salinity by newly hatched lobster larvae. *J. Fish. Res. Board Can.,* **24**: 1403–1406.

SHELFORD, V. E., 1915. Principles and problems of ecology as illustrated by animals. *J. Ecol.,* **3**: 1–23.

TURNER, H. J., JR., AND C. J. GEORGE, 1955. Some aspects of the behavior of the quahaug *Venus mercenaria* during the early stages. *8th Rep. Invest. Shellf., Mass. Dept. Nat. Res., Div. Mar. Fish., Commonwealth Mass.,* **8**: 5–14.

WELSH, J. H., 1932. Temperature and light as factors influencing the rate of swimming of the larvae of the mussel crab, *Pinnotheres maculatus* Say. *Biol. Bull.,* **63**: 310–326.

Reference: *Biol. Bull.,* **140**: 502–519. (June, 1971)

BEHAVIORAL AND ELECTROPHYSIOLOGICAL STUDIES OF HYDRA. II. PACEMAKER ACTIVITY OF ISOLATED TENTACLES[1]

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The tentacles of a coelenterate provide sensory surfaces for the transduction of stimuli from the animal's environment. Their primary role in the capture of prey is well known. Recent work with sea anemones, moreover, emphasizes their importance in complex behavior sequences such as swimming, and their responses to host organisms in commensal relationships (Hoyle, 1960; Robson, 1961a, 1961b; Josephson and March, 1966; Ross and Sutton, 1961).

In the literature at the turn of the century there were sporadic reports that isolated tentacles responded to mechanical and chemical stimuli much in the same way as they did when attached to the animal (Parker, 1896; Üxkull, 1909). Such observations were made long before the techniques of electrical recording from coelenterates became available. However, studies of the behavior of excised actinian tentacles prompted Parker to suggest that "each tentacle contained a neuromuscular mechanism sufficient for its own activity and it is therefore not dependent upon the nervous control of other parts of the animal's body for the production of those movements that it ordinarily exhibits" (Parker 1917, page 95).

Passano and McCullough (1962, 1964, 1965) were the first to record spontaneous electrical activity in *Hydra.* On the basis of such recordings they postulated several pacemaker systems whose interactions control the behavior of the animal. One set of potentials, associated with tentacle contraction, was recorded as small pulses from external electrodes placed on the body column. These Tentacle Contraction Pulses (TCP's) initiated from pacemakers located in the proximal part of each tentacle were observed to sometimes precede a burst of potentials from the Contraction Burst Pacemaker System. This system produced potentials associated with contraction of the hydra column (Passano and McCullough, 1963). The interactions among these two pacemaker systems are discussed in a subsequent paper (Rushforth, in preparation).

The present investigations stemmed from earlier observations that tentacles cut from hydra contracted spontaneously and in response to external stimuli (Rushforth, 1967b). This paper, the second in a series of reports on the behavior and electrical activity of *Hydra* describes patterns of spontaneous potentials in isolated tentacles and modifications of the patterns by external stimuli.

MATERIALS AND METHODS

The preparations used in these studies were isolated tentacles primarily from two species; *H. pirardi* and *H. pseudoligactis,* (possibly *H. canadensis*). The

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methods employed for culturing the hydra were previously described (Rushforth, 1971).

External electrical recordings were made from isolated tentacles using a glass suction electrode (Josephson, 1967). The electrode configuration and methods of recording have been discussed previously (Rushforth, 1971). Efforts to record from two sites on a single tentacle were unsuccessful, even when the electrodes used were flexible plastic tubing. Invariably one end of the tentacle would pull out of one of the electrodes during contraction. Thus, it was not possible to measure the conduction velocity of stimulated TCP's.

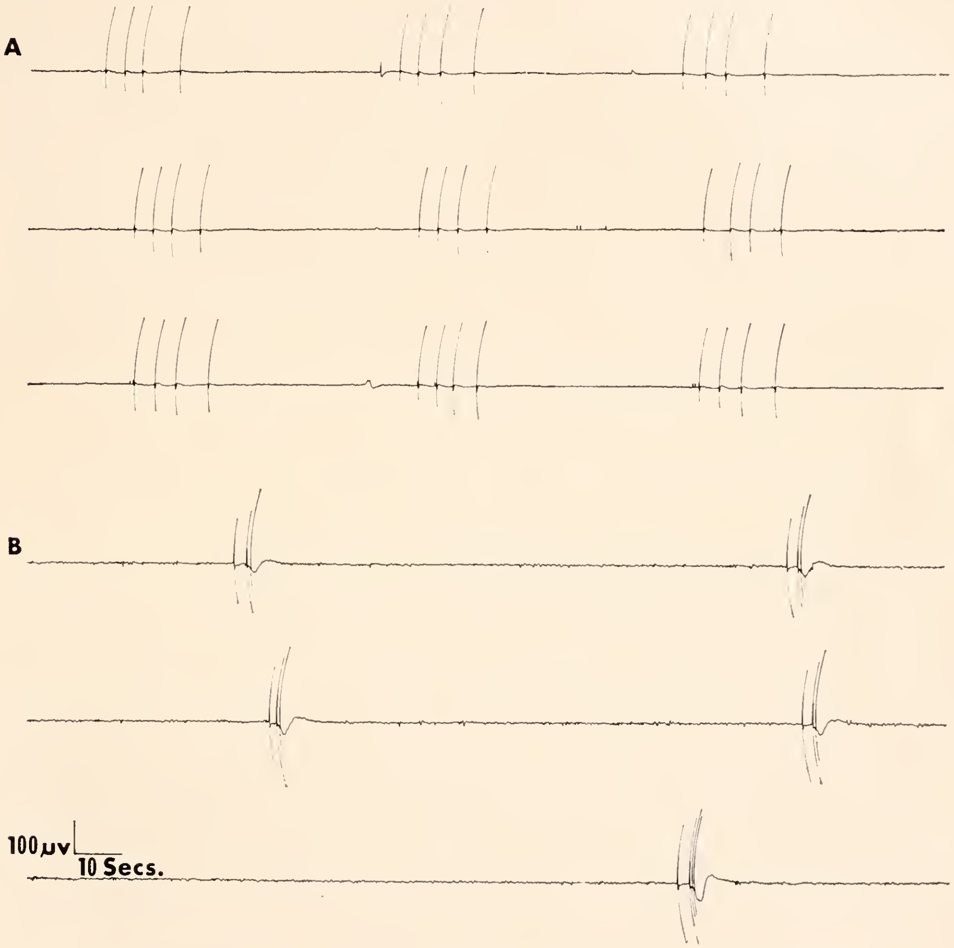

Figure 1. Spontaneous electrical activity recorded from isolated tentacles; bursts of TCP's from a *H. pirardi* (A) and a *H. pseudoligactis* (B).

Overt behavior of tentacles was observed with a dissecting microscope and recorded manually using telegraph keys attached to two channels of a penwriter.

Such observations were made at the same time as recordings of the electrical activity in order to make correlations between behavioral and electrical events. Statistical analyses were performed on the inter-TCP interval measurements taken from recordings of several hours duration using methods described previously (Rushforth, 1971).

A tentacle preparation was sometimes stimulated while its electrical activity and behavior were observed. Photic stimulation was administered using methods previously described (Rushforth, 1971). Specimens of *Artemia,* one at a time, were introduced into the medium above the tentacle to test the effect of live *Artemia salina* nauplii on the spontaneous TCP activity. If an *Artemia* struck the tentacle it sometimes became ensnarled by nematocysts and was thus captured. If the *Artemia* placement was made carefully, little mechanical disturbance was introduced to the preparation other than that due to the *Artemia* itself. Reduced glutathione of known concentrations were pipetted into the culture medium to determine the effect of this tripeptide on tentacle behavior and TCP frequency. The preparation was mechanically stimulated by agitating the water in the recording dish with a magnetic stirrer. Turning the stirrer on for brief periods gave pulses of mechanical agitation. Such pulses are observed as discrete increases in the noise level in the electrical record, but do not mask the TCP's emitted by the preparation.

RESULTS

Temporal patterns of spontaneously produced tentacle contraction pulses

(1) *Inter-pulse interval distributions.* The electrical activity of the isolated tentacles of both species consisted exclusively of large compound pulses, up to 10 mv in amplitude and of approximately 200 msec duration. Visual observations of the preparation indicated that the pulses were associated in a one-to-one fashion with symmetric contractions of the tentacle. These potentials therefore are TCP's, recorded directly from tentacles. They are much larger than TCP's observed in records taken with external electrodes placed on the base of the hydra body column. Records taken from the base of the isolated tentacle consisted primarily of bursts of TCP's. No potentials similar to RP's were observed. The typical burst patterns of TCP's are illustrated in Figure 1 for an isolated tentacle of *H. pirardi* (A), and *H. pseudoligactis* (B). The patterns of electrical activity taken from isolated tentacles are more homogeneous both within a single preparation and among the various tentacles of the same species, than those of the Column Contraction System (see Rushforth, 1971). However, there is variability in the numbers of pulses per burst, the interburst interval lengths and among the interval lengths between successive pulses in a burst.

Measurements of the interval lengths (to the nearest 0.4 second) between successive TCP's were made from records of two or three hours duration for isolated tentacles of *H. pirardi* and from records of approximately 8 hours duration for *H. pseudoligactis.* Plots of inter-pulse histograms and first order joint interval scatter diagrams were constructed for each preparation in the sample (*H. pirardi* $n=7$, *H. pseudoligactis* $n=5$). Figures 2 and 3 represent such plots for TCP's from a single isolated tentacle of *H. pirardi* and one from *H. pseudoligactis.* The histograms depicting the percentage of inter-TCP intervals in ten second classes constitute the lower graphs in the two figures. Both histograms are bimodal. Bimodality of the inter-pulse interval histograms and L shaped first order joint interval scatter diagrams (upper graphs) are characteristic of a pattern made up of bursts of pulses (Rushforth, 1971).

For both species the initial modal class of the lower graph includes intervals less than ten seconds in length and is, therefore, made up of intervals between pulses of a burst. These intervals comprise 76 and 72 per cent of all the intervals for the two respective records. A secondary modal class of 40–50 seconds for *H. pirardi* contains about 10 per cent of the intervals. These intervals are the inter-burst intervals. In the *H. pseudoligactis* preparation a relatively smaller secondary modal class occurs between 20 and 30 seconds, making up only 3–4 per cent of all interval lengths. A much larger percentage (65 per cent) of inter-pulse intervals longer than 10 seconds have lengths greater than a minute. The middle histograms depict the percentage distributions of those inter-TCP intervals that are less than 30 seconds. These two histograms show a marked

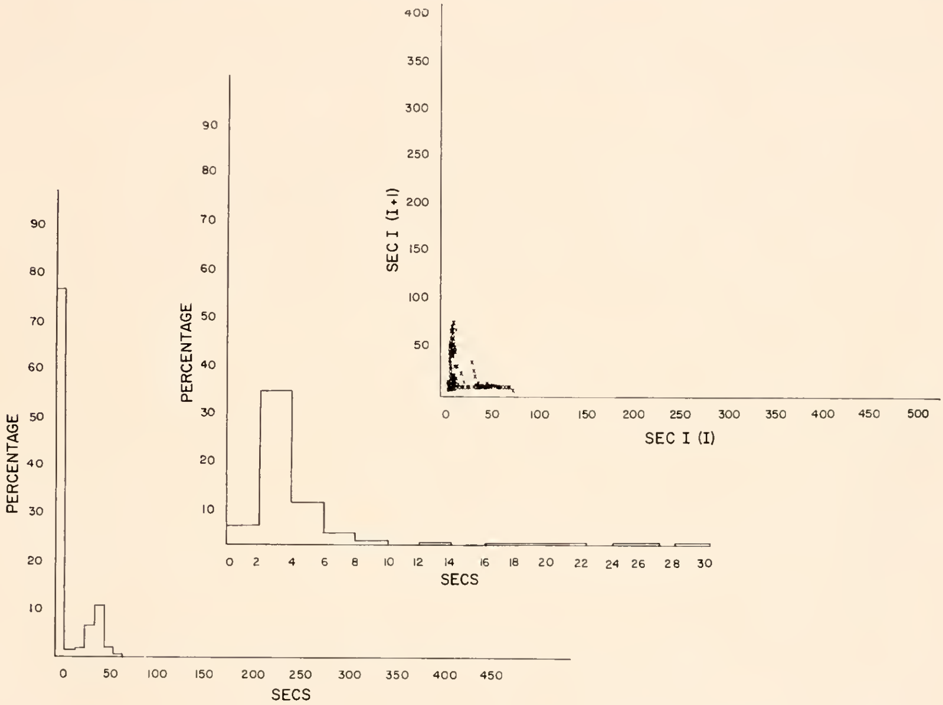

FIGURE 2. Inter-pulse interval distributions for TCP's from an isolated tentacle of *H. pirardi*. The lower histogram is a plot of the percentage of intervals falling in 10 second interval classes for all intervals in a four hour record. The middle histogram consists of intervals less than 30 seconds in length from this record. It is a plot of the percentage of intervals falling in two second classes. The upper graph is the first order joint interval scatter diagram for all intervals in the record.

difference in interval patterns for the two species. The distribution of interval lengths for *H. pirardi* is unimodal (Modal class 3–4 seconds) and roughly symmetrical, while in *H. pseudoligactis* most of the intervals are less than one second in length and the distribution is skewed to the right.

In both species the great majority of short intervals, those that occur within bursts, are less than 10 seconds. Ten seconds was therefore chosen as the cut-off point to classify intervals as either long or short intervals. A burst was defined as two or more pulses with successive inter-pulse interval lengths less than 10 seconds.

(2) *Inter-pulse interval measurements.* Calculations were made of various inter-pulse interval measurements for the samples of both species. These measurements are based on approximately two hour records from *H. pirardi* tentacles, and on approximately 8–12 hour recordings for the tentacles of *H. pseudoligactis.* The recording times were longer with *H. pseudoligactis* because of the lower frequency of TCP in this species. Mean values for the interval parameters together with their standard errors are given in Table I.

Table I

Measurements of intervals between tentacle contraction pulses for isolated tentacles of H. pirardi *and* H. pseudoligactis *(The long interval lengths and inter-burst interval lengths differ because occasionally two or more long intervals occured between bursts)*

Measurement	Mean (Standard error)	
	H. pirardi (n = 7)	*H. pseudoligactis* (n = 5)
Number of intervals/12 hr	3565 (806)	1075 (200) $P < 0.001$
Short interval lengths (sec)	3.6 (0.3)	2.0 (0.4) $P < 0.05$
Long interval lengths (sec)	41.5 (5.8)	194.1 (31.6) $P < 0.01$
Per cent of intervals classified as long intervals	25.4 (0.7)	25.9 (1.5) $P > 0.10$
Number of intervals per burst	3.1 (0.1)	3.7 (0.2) $P < 0.05$
Mean interval length per burst (sec)	3.7 (0.1)	1.9 (0.4) $P < 0.01$
Burst length (sec)	11.4 (0.9)	7.4 (1.9) $P > 0.05$
Inter-burst interval length (sec)	42.2 (5.6)	208.5 (26.1) $P < 0.001$

Several marked differences between the patterns of spontaneously produced TCP's in the two species are evident from Table I. The TCP frequencies, as measured by the numbers of TCP intervals/12 hours, are significantly greater ($P < 0.001$) for *H. pirardi* than for *H. pseudoligactis.* The interval lengths of short intervals (those less than 10 seconds in duration) are significantly ($P < 0.01$) longer for *H. pirardi.* This gives rise to a significantly longer mean interval length for the intervals within bursts ($P < 0.01$) for this species. However, there are more intervals within a burst for *H. pseudoligactis* ($P < 0.05$). The net effect of these two factors results in approximately the same burst lengths for the two species. The lengths of the long intervals (those greater than 10 seconds) are much greater in *H. pseudoligactis*, reflected in a significantly greater mean inter-burst interval ($P < 0.01$). However, the percentages of all intervals which are greater than 10 seconds in length are very similar for all tentacles in both species.

(3) *Lengths of short intervals in a TCP burst.* With CP bursts recorded from the column the inter-pulse intervals first become smaller and then lengthen again toward the end of the burst (Passano and McCullough, 1963, Rushforth, 1971). The intervals within TCP bursts from isolated tentacles behave somewhat similarly.

In Table II, the mean values of interval lengths for bursts having differing numbers of intervals are presented as a function of the order of the interval within a burst. For *H. pirardi*, the mean values are obtained by pooling the bursts for a sample of seven preparations of this species. For *H. pseudoligactis*, mean values are based on bursts pooled from five preparations. Based on an anlysis of variance test, mean interval lengths are significantly different ($P < 0.01$) among the various intervals in the bursts. This result applies in all cases except for bursts consisting of two intervals in *H. pirardi* isolated tentacles. In general, the second interval in a burst is shorter than the first interval, while succeeding intervals systematically increase in length. In every case, except bursts of two intervals for *H. pseudoligactis*, the last interval is longer than the other intervals in the burst. This consistent pattern is observed in spite of the previously described inter-species differences in the activities of the Tentacle Contraction Pacemaker System.

TABLE II

Relationship between interval length and position of the interval in a burst for isolated tentacles of H. pirardi *and* H. pseudoligactis

Number of short intervals per burst	Mean interval (sec) Position of interval in the burst						Number of bursts
	1	2	3	4	5	6	
A *H. pirardi*							
1	5.3						45
2	3.5	3.7					273
3	3.0	2.6	4.4				1049
4	2.8	2.3	3.5	5.5			346
5	2.7	2.4	3.2	4.2	4.5		48
B *H. pseudoligactis*							
1	2.4						119
2	2.5	1.2					188
3	1.2	1.1	1.8				274
4	1.1	0.7	1.2	4.0			160
5	1.2	0.9	1.3	3.1	4.4		84
6	2.2	1.0	1.0	2.5	2.9	4.0	35

(4) *Temporal pattern of contraction pulses from segments of a tentacle.* The entire structure of the excised tentacle is not required for the production of TCP's. Bursts of potentials may be recorded from segments cut from a tentacle. The patterns of TCP firing from such preparations are quite similar to those of

the entire isolated tentacle. For example, a distal segment, approximately $\frac{1}{5}$ the length of the whole *H. pseudoligactis* tentacle, gave histograms and a first order joint scatter diagram very similar to those of an intact isolated tentacle of this species (Fig. 4). The inter-pulse histograms are bimodal and the first order joint interval scatter diagram gives the L-shaped plot characteristic of bursts of potentials. Thus, TCP distributions from segments of a tentacle are similar to those of the entire tentacle.

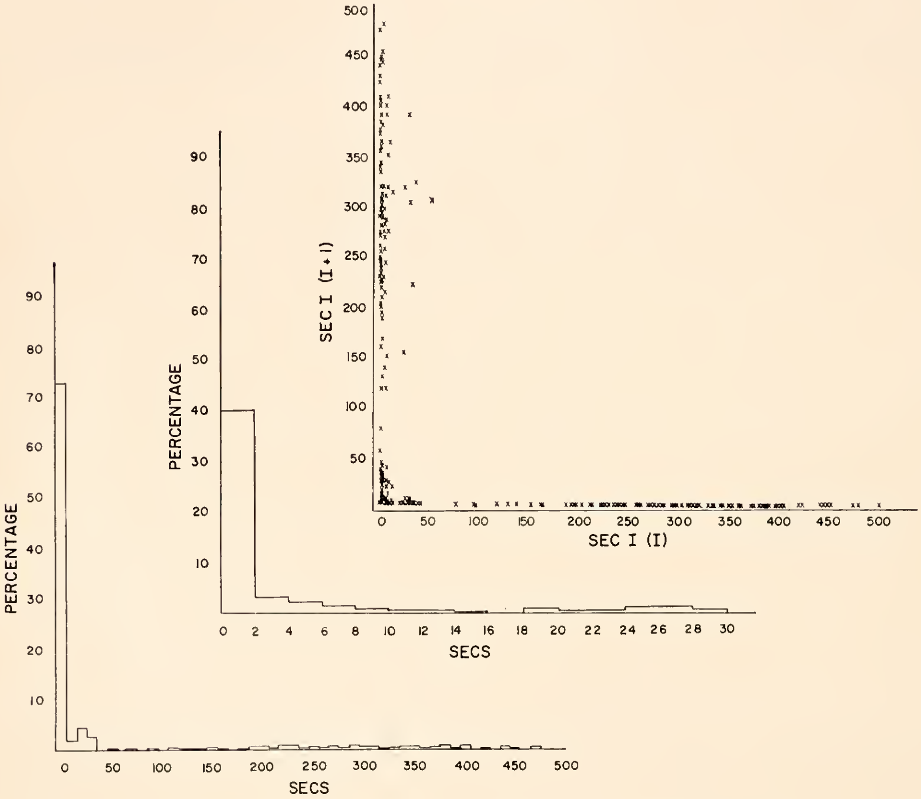

FIGURE 3. An inter-pulse interval representation like that of Figure 2 for *H. pseudoligactis*. The data is from a 12 hour continuous recording.

Changes of TCP activity with external stimulation

(1) *Effects of light stimulation.* The TCP activity patterns are changed when isolated tentacles of *H. pirardi* and *H. pseudoligactis* are exposed to a regime of two minute periods of direct illumination interspersed with two minute periods of ambient light. In both species, TCP's are more frequent during the strong light periods. This is consistent with the finding that direct illumination increases the TCP frequency from attached tentacles in the two species, (Rushforth, in preparation). Examples of the electrical records of isolated tentacles exposed to such light

stimulation are given in Figure 5, (A. *H. pirardi*; B. *H. preudoligactis*). It is seen that for three sequences, consisting of two minutes of direct light followed by two minutes of ambient light, TCP pulses are more numerous in the periods of direct illumination.

Features of TCP activity in both periods of direct and ambient illumination are summarized in Table III. The pulse frequency, burst frequency, and number of intervals per burst are all significantly greater ($P < 0.01$) in the periods of direct illumination. However, there is no significant difference ($P > 0.10$) in the mean interval/burst. The same systematic relationship between the interval length and its position in the burst, noted previously for spontaneously produced bursts of TCP's, was observed for light stimulated tentacles. For both species, intervals are shorter in mid-burst than at the start or end of the burst.

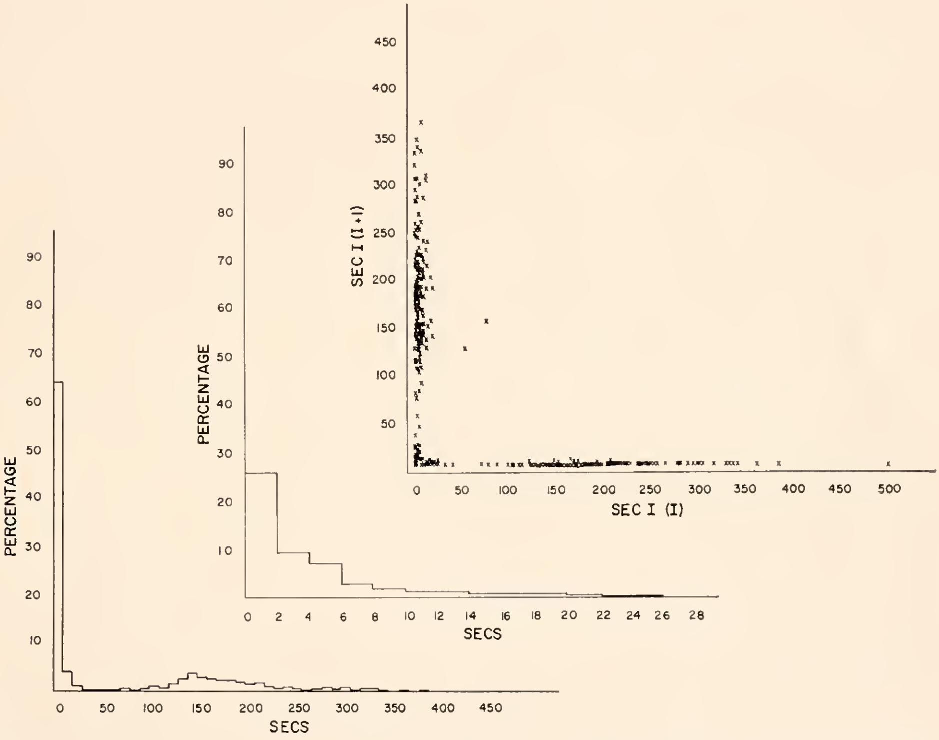

FIGURE 4. An inter-pulse interval representation like that of Figure 2 for a small distal portion of a *H. pseudoligactis* tentacle. The data is from a 12 hour recording.

In order to verify that direct illumination of an isolated tentacle increases the number of pulses within a burst, preparations were exposed to strong light at different points in the burst-inter-burst cycles. Preparations of *H. pirardi* were illuminated directly following the first pulse in a spontaneously produced burst.

At other times the preparations were exposed to strong light at random points in time and the light was turned off following the first pulse in the light-induced burst. The numbers of pulses/burst and the mean interval/burst for bursts stimulated by these two methods, are compared with "control" bursts. The groups of control bursts were composed of bursts immediately preceding and immediately following both types of light modulated bursts. Comparisons were based on 20–50 bursts for both types of stimulation for five individual tentacles. It was found that the number of pulses per burst was significantly increased ($P < 0.01$) when the preparation was exposed to strong light after the first pulse in a spontaneous burst (mean = 5.4, S.E. = 0.1). Conversely, the number of pulses per burst is significantly decreased ($P < 0.01$) when light is removed after the first pulse in a light induced burst (mean = 2.9, S.E. = 0.1). These values were compared to the average values of control bursts immediately preceding and following light stimulation (mean = 3.9, S.E. = 0.1). The two modes of light stimulation do not significantly ($P > 0.10$) affect the mean interval length/burst.

TABLE III

Effects of light on TCP bursts in isolated tentacles of H. pirardi *and* H. pseudoligactis

	Mean (S.E.) based on five preparations for each species		
	Direct illumination	Ambient illumination	
a. *H. pirardi*			
Number of TCP's/2 min	13.6 (0.6)	9.0 (0.6)	$P < 0.01$
Number of bursts/2 min	2.9 (0.1)	2.2 (0.1)	$P < 0.01$
Number of intervals/burst	3.7 (0.1)	3.1 (0.1)	$P < 0.01$
Mean interval/burst	3.5 (0.1)	3.5 (0.1)	$P > 0.10$
b. *H. pseudoligactis*			
Number of TCP's/2 min	11.3 (0.6)	6.2 (0.9)	$P < 0.01$
Number of bursts/2 min	2.3 (0.1)	1.4 (0.2)	$P < 0.01$
Number of intervals/burst	3.9 (0.1)	3.4 (0.1)	$P < 0.05$
Mean interval/burst	2.0 (0.2)	1.8 (0.1)	$P > 0.10$

In addition, in both cases intervals are shorter in mid-burst than at the start or end of the burst.

(2) *Effects of mechanical stimulation.* Single TCP's can be induced with weak mechanical stimuli to isolated tentacles and bursts of TCP's can be initiated with stimuli of greater intensity (Fig. 6). At high stimulus frequencies (greater than 1/15 sec) the response to mechanical stimulation rapidly adapts. Induction of the TCP system to fire as single pulses, using mechanical stimulation, is associated with inhibition of TCP bursts. However, with cessation of mechanical stimulation, the normal spontaneous burst pattern of TCP production is soon restored (Fig. 6).

(3) *Effects of feeding stimuli.* Specimens of *Hydra* undergo complex feeding reactions following the capture of a prey organism by nematocyst discharge from the tentacles. The following experiments were undertaken to determine the effects on the behavior and electrical activity of isolated tentacles of stimuli which elicit feeding reactions in hydra.

Initial behavioral observations made in the absence of electrical recordings indicated that placement of a single *Artemia salina* nauplius on an isolated tentacle of *H. pseudoligactis* inhibited the normal spontaneous contraction burst activity.

While bursts of longitudinal contractions were suppressed, the tentacle underwent a considerable number of asymmetric contractions. These contractions were very similar to the writhing activity frequently observed in the tentacles of an intact hydra during the middle stages of the feeding response. Invariably after several minutes of writhing activity the specimen of *Artemia* would detach from the tentacle and fall inertly to the bottom of the dish. The tentacle's contractile behavior gradually changed from uncoordinated contractions to the normal contraction burst activity. The behavioral sequence just described may be elicited equally well with aqueous extracts of *Artemia salina* or by administering reduced glutathione in concentrations of 10^{-5} *M* GSH to the isolated tentacles.

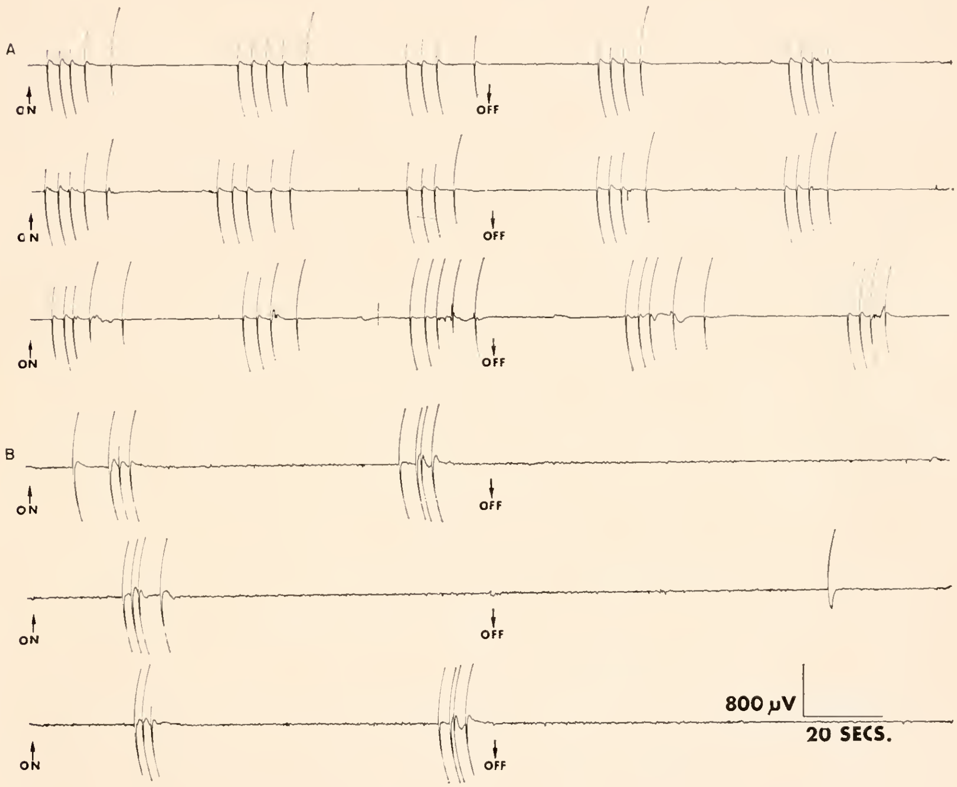

FIGURE 5. Effects of light stimulation on TCP firing. Frequency of bursts of TCP's in two minute periods of direct illumination (on to off) compared with the frequency in ambient light, for isolated tentacles *H. pirardi* (A, upper three records) and *H. pseudoligactis* (B, lower three records).

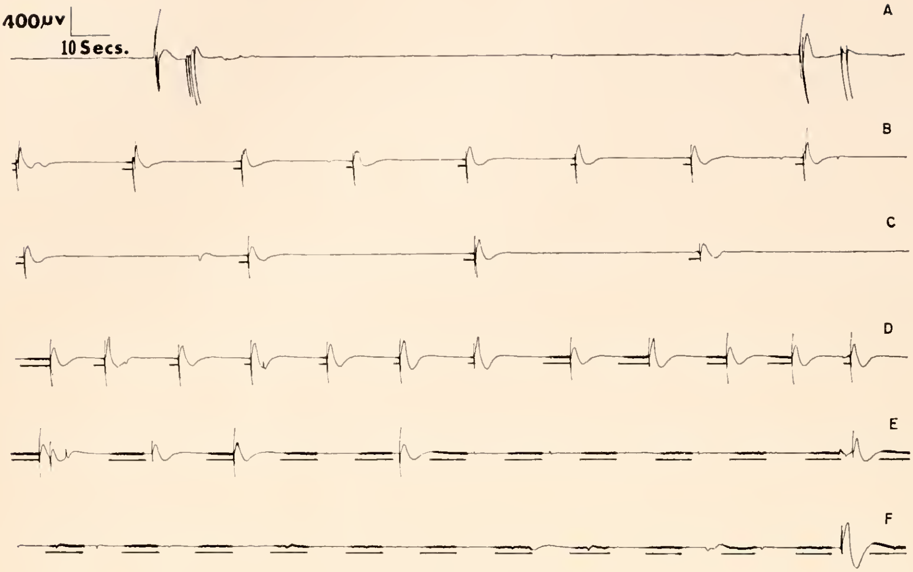

FIGURE 6. Effects of mechanical stimulation on TCP firing in an isolated tentacle of *H. pseudoligactis*. Spontaneous bursts of TCP's (A) and single TCP's induced by agitation of the surrounding culture medium (B, C, D, E, and F). The increase in the noise level and the unbroken lines below each electrical record show the duration of stimulation. Adaptation of the preparation to mechanical stimulation occurs in E. and F.

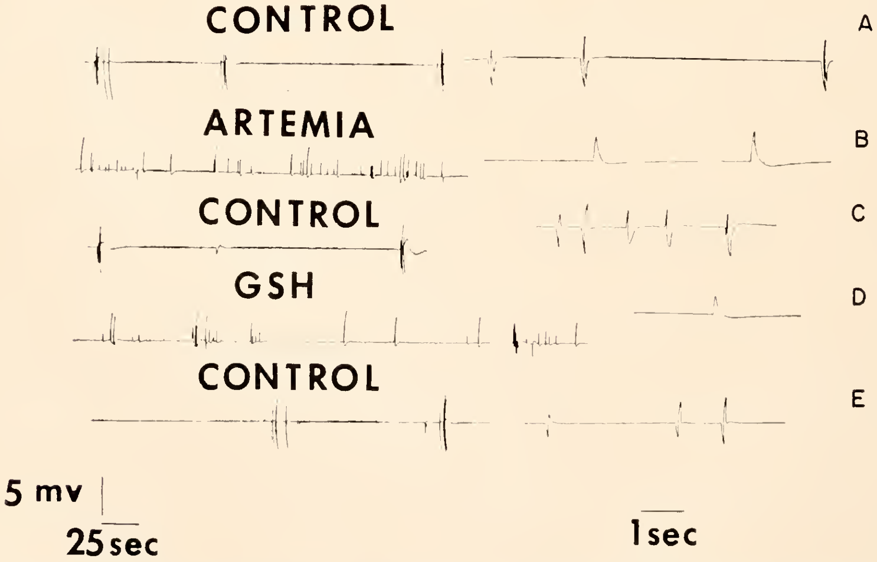

FIGURE 7. Effects of *Artemia salina* nauplii and 10^{-5} *M* GSH on the electrical activity of an isolated *H. pseudoligactis* tentacle. Records on the right hand side of the figure were

The records in Figure 7 show the effects of an *Artemia salina* nauplius and 10^{-5} *M* GSH on the electrical activity of an isolated *H. pseudoligactis* tentacle. The short sequences in the right hand portions of each record were taken at a higher recording speed to show the shapes of the pulses. Monophasic pulses associated with tentacle writhing supplant the normal pattern of spontaneously produced multiphasic TCP's. During the initial phases of the response these pulses are relatively frequent (every 1–3 sec). They occur primarily as single events, and have lower amplitude than TCP's, usually no greater than one millivolt. Gradually, however, TCP's return to the record, sometimes first as single pulses and then later as bursts. The monophasic pulses decrease in frequency and the burst pattern is completely restored. This follows release of the nauplius from the tentacle, although no abrupt change in the electrical activity of the preparation is correlated with the event of prey release. In the case of 10^{-5} *M* GSH the TCP burst pattern usually returns within 15–20 minutes after addition of GSH.

Inhibition of TCP bursts on capture of a single *Artemia* is reflected by significantly lengthened ($P < 0.01$) inter-burst interval. The inter-burst interval length is 3–5 fold greater than pre-stimulation values. After prey release, however, the subsequent ten inter-burst intervals are significantly smaller ($P < 0.01$) than those of corresponding inter-burst intervals preceding stimulation, suggesting post inhibitory excitation of the TCP pacemaker system in producing bursts.

DISCUSSION

(1) *Spontaneously produced TCP's.* Isolated tentacles produce bursts of potentials, while tentacles attached to the hydra produce both single pulses and bursts (Rushforth, in preparation). Surgical removal of the tentacles from the animal presumably excites the TCP system, and placing the wounded preparation in the suction electrode puts it in a stressful recording situation. The CP system of *H. pseudoligactis*, which normally produces single pulses, is induced to give bursts of potentials when exposed to increasing basal pressure in a suction electrode (Rushforth, 1971). Thus, the initial TCP burst pattern may reflect prior wounding and electrode suction. However, sequences of bursts have been recorded continuously from a tentacle for more than 24 hours after removal from the animal, long after wound healing and adaptation to the electrode should have taken place. Thus, the TCP burst pattern may reflect the removal of the electrical input from other pacemakers, rather than excitation from the initial wounding of the preparation.

In both species investigated the pattern of TCP's from isolated tentacles consisted of bursts of potentials. As with the Column Contraction System, however, the temporal characteristics of TCP's are quite different in *H. pirardi* and *H. pseudoligactis*. TCP frequencies are much greater for *H. pirardi* as are the interval lengths within a burst. However, this species has fewer pulses per

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taken at a higher recording speed to show the shapes of the pulses; A. spontaneous bursts of TCP's; B. monophasic pulses observed when the tentacle writhes after capturing an *Artemia;* C. restoration of the normal burst pattern of TCP's after the artemia has been released and writhing has terminated (30 minutes after B was taken); D. monophasic pulses induced by 10^{-5} *M* GSH (5 minutes after C was recorded); E. normal TCP burst pattern 30 minutes after the addition of GSH.

burst and significantly shorter inter-burst intervals than *H. pseudoligactis.* If the mean interval per burst and the inter-burst interval are plotted, points derived for the TCP patterns of the two species, form two non-overlapping sets (Fig. 8).

The entire structure of the isolated tentacle is not necessary for the production of the characteristic burst pattern of TCP's. Small blocks of tissue from the tentacles give patterns representative of the species. This suggests that potential pacemakers occur in different parts of a tentacle and that they all have similar properties.

(2) *Modifications in TCP burst patterns with external stimuli.* The effect of strong light stimulation increases the frequency of TCP's in isolated tentacles of both species. Both the frequency of bursts and the number of intervals per burst are increased by such stimulation. However, the mean interval length is unchanged as is the systematic relationship between the interval length and its position in the burst, found in spontaneously produced bursts of TCP's. Light stimulation at different points in the burst-inter-burst cycle may be used to add additional pulses to bursts or subtract pulses from the normal burst size. Thus, light can effect both mechanisms determining the occurrence of bursts and those controlling the production of pulses within the bursts.

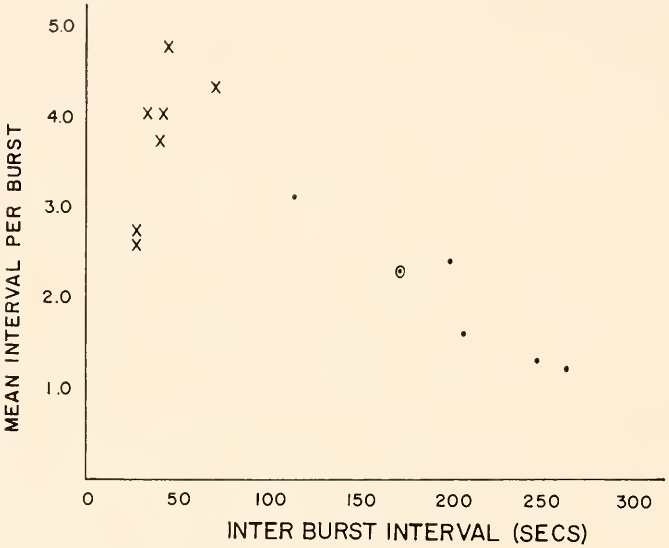

FIGURE 8. Mean interval per burst and inter-burst interval length for isolated tentacles of *H. pirardi* (crosses) and *H. pseudoligactis* (closed circles). The large closed circle represents values for a distal segment of tissue cut from an isolated tentacle of *H. pseudoligactis.*

Previous studies have shown that the tentacles are controlling factors in the response of the intact hydra to mechanical stimulation (Rushforth, 1965). When all the tentacles are removed the animal does not contract to such stimulation, suggesting that the initiation of mechanically induced contractions resides in the tentacle structure. In the present study it has been shown that isolated tentacles respond to mechanical agitation, changing their pattern of bursts of potentials to one of single pulses synchronized with the mechanical stimuli. As in the case of the whole animal this preparation adapts to repeated stimulation. With such adaptation the normal pattern of bursts of TCP's is restored. Adaptation of tentacles to mechanical stimulation and the associated changes in electrical activity may underlie the processes of habituation of the whole animal found on repeated stimulation (Rushforth, 1967a).

Inhibition of TCP bursts in isolated tentacles is also found with feeding stimuli. Asymmetric contraction potentials are elicited by live specimens of *Artemia*, aqueous extracts of *Artemia*, or the tripeptide reduced glutathione. The effects of these stimuli in producing feeding responses has been extensively studied by Lenhoff (1961, 1968). The present studies show an effect of the stimuli on an isolated pacemaker system in the animal.

The mechanisms underlying the production of the pulses associated with asymmetric movements comprising the uncoördinated writhing activity of isolated tentacles are unknown. While occasionally isolated pulses will be observed in the record of the unstimulated tentacle, their appearance on exposure to feeding stimuli is quite dramatic. There is insufficient evidence to indicate that they are produced by a discrete pacemaker system separate from the TCP System. It is possible that the pulses result from chemostimulatory effects on the TCP System, causing alterations in firing locii and the spread of conduction to neighboring pacemaker units. The net effect may be to partially block the normal mode of firing of the pacemaker system. Indirect evidence for this idea comes from the fact that their appearance is always associated with inhibition of bursts of TCP's. In absence of direct evidence, however, this remains purely a conjecture.

On first observation it was thought that the monophasic pulses might be associated with the coordinated "concert" movements of the hydra tentacles, which are characteristic of the feeding response. Concert activity has been found to be increased by feeding stimuli (Rushforth and Hofman, 1966). The monophasic pulses which were recorded were accompanied by writhing rather than smooth orally directed sweeping concert movements. Passano and McCullough (1963) observed that tentacle writhing was frequently a component of the light response of intact hydras. Such behavior was not accompanied by concerts, thus emphasizing the separateness of writhing and concert activity. Experiments with isolated tentacles show that after inhibition of TCP bursts by feeding stimuli there is increased excitability of the TCP burst system. This post inhibitory excitability has been observed with feeding stimuli in the case of the CP System (Rushforth, in preparation) and is reminiscent of processes of post inhibitory rebound seen in neural systems (Erlanger and Glasser, 1937).

(3) *Models of pacemaker discharge.* Several types of central neurons fire in clusters of spikes, separated by long intervals. Such bursts of potentials have been described in such diverse preparations as: mammalian hippocampal cells (Kandel and Spencer, 1961); locust flight motoneurons (Wilson, 1964); lobster cardiac ganglion neurons (Maynard, 1955); interneurons in the abdominal cord of the crayfish (Kennedy and Takeda, 1965); crustacean motoneuron preparations (Ikeda and Wiersma, 1964; Gillory and Kennedy, 1969, Davis, 1969); and the

"parabolic burster" cell of *Aplysia* (Strumwasser, 1965). Frequently the pattern of such bursts has been shown to be endogenous to the cell and not due to periodic synaptic input. Mechanisms of super-excitability after an impulse (Hodgkin, 1948, Wright and Coleman, 1964), depolarizing after-potentials (Kandel and Spencer, 1961, Kennedy and Mellon, 1964), and periodic changes in the conductivity for specific ions (Strumwasser, 1968) have been suggested for the production of such patterned discharge.

During the past five years considerable interest has arisen from the development of models describing the firing patterns of isolated neurons (Geisler and Goldberg, 1966, Junge and Moore, 1966, Calvin and Stevens, 1967, Stein, 1967). A common feature of such models is the specification that a pulse is generated when some measure of cell excitability, for example the membrane potential, reaches some critical threshold level. In most models the threshold is a time dependent function containing parameters of absolute and relative refractory periods. All of the models have the basic assumption of random elements in the pulse generating processes. Models specifically constructed to describe bursts of potentials have been described by Strumwasser, (1968) and Davis, (1969). Such models for bursts incorporate the basic features of the simpler models for single pulses, but assume additional components. In the case of bursts of potentials from swimmeret motoneurons in the lobster, the model assumes sinusoidal excitation to motoneuron units. This model has been successfully used in computer simulation studies to reproduce the temporal characteristics of observed discharge patterns (Davis and Murphy, 1969).

Quantification of the pulse patterns places some limits on the kinds of models which might be proposed to generate such patterns. A successful model must account for the following features: (1) there must be a mechanism to initiate and to terminate bursts. The termination of bursts may be a result of negative feedback as has been proposed in a model for the CP System (Rushforth, 1971); (2) short term changes in firing parameters must be included to account for variability in the number of pulses per burst, and in the inter-burst interval length. Such parameter changes must be consistant with the modifications in burst-interburst characteristics effected by light stimulation, *i.e.*, simultaneous increase in pulse number and reduction in interburst interval length; (3) initial decrease and later increase in pulse intervals within the burst, seen in both unstimulated TCP's and those induced by light, must be simulated by the discharge patterns derived from the model. This relationship is a constant feature of both TCP and CP bursts, and has been observed in other hydroids (Morin, 1969), and in other inverterbrate systems (Strumwasser 1965, Davis 1969). This functional relationship was the restriction giving rise to the assumption of sinusoidal input in the model of bursts for swimmeret motoneurons in lobsters (Davis and Murphy, 1969).

Our experiments suggest that while the number of pulses per burst and the inter-burst interval length are quite variable components of TCP discharge patterns, the mean interval per burst and the ordered lengths within a burst are constant properties of the output. Neither can easily be experimentally changed even using patterned stimulation.

The results of these studies strongly demonstrate the similarity of the TCP System in isolated tentacles to that of the CP System of the hydra body column, particularly in the case of *H. pirardi*. Temporal characteristics of spontaneously produced bursts of pulses and their modification with external stimuli, are very similar for the two systems. It seems quite likely that the mechanisms of pulse production for TCP's are the same as those of CP generation in the body column. The similar size of the potentials, their duration and compound shape together with the temporal characteristics all point to similar mechanisms of production. Thus the model originally suggested for the CP System (Rushforth, 1971) might be an appropriate one for the TCP System.

In this paper we have described the temporal characteristics of spontaneously produced electrical potentials from an isolated hydroid pacemaker system and their modification with external stimuli. In future studies, isolated pacemaker systems in other hydroids will be investigated to determine the generality of the features observed here for this preparation. In addition we will investigate the interactions between different pacemaker systems. By such means we hope to gain insight into the coordinating mechanisms controlling behavior in coelenterates, the most simply organized living animals with an indisputable nervous system.

SUMMARY

1. Electrical potentials associated with coordinated contraction of the longitudinal muscles can be recorded from isolated tentacles of *Hydra*. In both *H. pirardi* and *H. pseudoligactis* these Tentacle Contraction Pulses (TCP's) occur chiefly in bursts, which are more frequent in *H. pirardi*. The bursts are approximately the same length in the two species but those of *H. pirardi* contain more pulses.

2. Inter-pulse interval lengths within a burst decrease but then lengthen again toward the end of the burst. Small segments of a tentacle produce the same pulse patterns as whole tentacles, indicating that there are a number of potential pacemakers with similar properties dispersed within a tentacle.

3. Strong illumination increases the burst frequency and the number of pulses per burst. Pulses of mechanical stimuli induce single TCP's and inhibit TCP bursts. Isolated tentacles habituate to repeated mechanical stimuli. Exposure of the tentacle to live or aqueous extracts of *Artemia* nauplii, or 10^{-5} *M* GSH inhibits TCP bursts and monophasic pulses are induced which are associated with asymmetric writhing movements.

LITERATURE CITED

Calvin, W. H., and C. F. Stevens, 1967. Synaptic noise as a source of variability in the interval between action potentials. *Science*, **155**: 842–844.

Davis, W. J., 1969. The neural control of swimmeret beating in the lobster *J. Exp. Biol.*, **50**: 99–117.

Davis, W. J., and R. K. Murphy, 1969. Discharge patterns of swimmeret motoneurones in the lobster, simulated with a digital computer. *J. Exp. Biol.*, **50**: 119–128.

Erlanger, J., and H. S. Glaser, 1937. Electrical signs of nervous activity. University of Pennsylvania Press, Philadelphia, 221 pp.

Geisler, C. D., and J. M. Goldberg, 1966. A stochastic model of repetitive activity of neurons. *Biophys. J.*, **6**: 53–69.

GILLORY, H. L., AND D. KENNEDY, 1969. Pattern generation in a crustacean motoneuron. *J. Neurophysiol.*, **32**: 595–606.

HOYLE, G., 1960. Neuromuscular activity in the swimming sea-anemone, *Stomphia coccinea* (Muller). *J. Exp. Biol.*, **37**: 671–688.

HODGKIN, A. L., 1948. The local electrical changes associated with repetitive action in a non-modullated axon. *J. Physiol.*, **107**: 165–181.

IKEDA, K., AND C. A. G. WIERSMA, 1964. Autogenic rhythmicity in the abdominal ganglia of the crayfish: the control of swimmeret movements. *Comp. Biochem. Physiol.*, **12**: 107–115.

JOSEPHSON, R. K., 1967. Conduction and contraction in the column of hydra. *J. Exp. Biol.*, **47**: 179–190.

JOSEPHSON, R. K., AND S. C. MARCH, 1966. The swimming performance of the sea-anemone Boloceroides. *J. Exp. Biol.*, **44**: 493–506.

JUNGE, D. AND G. P. MOORE, 1966. Inter spike-interval fluctuations in *Aplysia* pacemaker neurons. *Biophys. J.*, **6**: 411–434.

KANDEL, E. R. AND W. A. SPENCER, 1961. Electrophysiology in hippocampal neurons. II. Afterpotentials and repetitive firing. *J. Neurophysiol.*, **24**: 243–259.

KENNEDY, D. AND DEF. MELLON, 1964. Synaptic activiation and receptive fields in crayfish interneurons. *Comp. Biochem. Physiol.*, **13**: 275–300.

KENNEDY, D. AND K. TAKEDA, 1965. Reflex control of abdominal flexor muscles in the crayfish. II. The tonic system. *J. Exp. Biol.*, **43**: 229–246.

LENHOFF, H. M., 1961. Activation of the feeding reflex in *Hydra littoralis*. Pages 203–232. in H. M. Lenhoff and F. W. Loomis, Eds., *The Biology of Hydra and Some Other Coelenterates.* University of Miami Press, Coral Gables, Florida.

LENHOFF, H. M., 1968. Chemical perspectives on the feeding response, digestion and nutrition of selected coelenterates, Pages 158–221 in B. Scheer and M. Florkin, Eds., *Chemical Zoology, Vol. II.* Academic Press, New York.

MAYNARD, D. M., 1955. Activity in a crustacean ganglion. II. Pattern and interaction in burst formation. *Biol. Bull.*, **109**: 420–436.

MORIN, J. G., 1969. Mechanisms controlling behavior and bioluminescence in the colonial hydroid *Obelia. Ph.D thesis, Harvard University*, Cambridge, Massachusetts, 156 pp.

PARKER, G. H., 1896. The reactions of Metridium to food and other substances. *Bull. Mus. Comp. Zool.*, **29**: 107–119.

PARKER, G. H., 1917. The movements of the tentacles in Actinians. *J. Exp. Zool.*, **22**: 95–110.

PASSANO, L. M., AND C. G. MCCULLOUGH, 1962. The light response and rhythmic potentials of Hydra. *Proc. Nat. Acad. Sci.*, **48**: 1376–1382.

PASSANO, L. M., AND C. B. MCCULLOUGH, 1963. Pacemaker hierarchies controlling the behavior of hydras. *Nature*, **199**: 1174–1175.

PASSANO, L. M. AND C. B. MCCULLOUGH, 1964. Coordinating systems and behavior in *Hydra*. I. Pacemaker system of the periodic contractions. *J. Exp. Biol.*, **41**: 643–664.

PASSANO, L. M., AND C. B. MCCULLOUGH, 1965. Coordinating systems and behaviour in *Hydra*. II. The rhythmic potential system. *J. Exp. Biol.*, **42**: 205–231.

ROBSON, E. A., 1961a. Some observations on the swimming behavior of the anemone *Stomphia coccinea*. *J. Exp. Biol.*, **38**: 343–363.

ROBSON, E. A., 1961b. The swimming response and its pacemaker system in the anemone *Stomphia coccinea*. *J. Exp. Biol.*, **38**: 685–694.

ROSS, D. M., AND L. SUTTON, 1961. The response of the sea anemone *Calliactis parasitica* to shells of the hermit crab *Pagurus barnhardus*. *Proc. Roy. Soc. London Series B.*, **155**: 266–281.

RUSHFORTH, N. B., 1965. Behavioral studies of the coelenterate *Hydra pirardi* Brien. *Anim. Behav.*, Supp. **1**: 30–42.

RUSHFORTH, N. B., 1967a. Chemical and physical factors affecting behavior in *Hydra*: interactions among factors affecting behavior in *Hydra*. Pages 369–390 in W. C. Corning and S. C. Ratner, Eds., *Chemistry of Learning.* Plenum Press, New York.

RUSHFORTH, N. B., 1967b. Behavioral responses of isolated tentacles of *Hydra*. *Biol. Bull.*, **133**: 449.

RUSHFORTH, N. B., AND F. HOFMAN, 1966. Behavioral sequences in the feeding response of *Hydra littoralis*. *Biol. Bull.*, **131**: 403–404.

RUSHFORTH, N. B., 1971. Behavioral and electrophysiological studies of *Hydra*: I. An analysis of contraction pulse patterns. *Biol. Bull.*, **140**: 255–273.

STEIN, R. B., 1967. Some models of nueronal variability. *Biophys. J.*, **7**: 37–68.

STRUMWASSER, F., 1965. The demonstration and manipulation of a circadian rhythm in a single neuron. Pages 444–462 in J. Aschoff, Ed., *Circadian Clocks*. North Holland Publishing Co., Amsterdam.

STRUMWASSER, F., 1968. Membrane and intracellular mechanisms governing endogenous activity in neurons. Pages 329–341 in F. D. Carlson, Ed., *Physiological and Biochemical Aspects of Nervous Integration*. Prentice-Hall, Englewood Cliffs, New Jersey.

ÜXKULL, J. V., 1909. *Unwelt und Innerwelt der Tiere*. J. V. Springer, Berlin, 259 pp.

WILSON, D. M., 1964. Relative refractoriness and patterned discharge of locust flight motor neurons. *J. Exp. Biol.*, **41**: 191–205.

WRIGHT, E. B., AND P. D. COLEMAN, 1954. Excitation and conduction in crustacean single motor axons. *J. Cell Comp. Physiol.*, **43**: 133–164.

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1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

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